TUMOUR GLYCOSYLATION AND GLYCOPROTEINS AS BIOMARKERS IN COLORECTAL CANCER

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ACADEMIC DISSERTATION

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Kaprio T, Fermér C, Hagström J, Mustonen H, Böckelman C, Nilsson O, Haglund C. Podocalyxin is a marker of poor prognosis in colorectal cancer. BMC Cancer, 14:493, 2014

II

Kaprio T, Hagström J, Fermér C, Mustonen H, Böckelman C, Nilsson O, Haglund C. A comparative study of two PODXL antibodies in 840 colorectal cancer patients. BMC Cancer, 14:494, 2014

III

Kaprio T, Satomaa T, Heiskanen A, Hokke C.H, Deelder A.M, Mustonen H, Hagström J, Carpen O, Saarinen J, Haglund C. N-glycomic profiling as a tool to separate rectal adenomas from carcinomas. Mol Cell Proteomics, 2015 Feb;14(2):277-88

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Kaprio T, Hagström J, Mustonen H, Andersson L, Haglund C. REG IV independently predicts better prognosis in non-mucinous colorectal cancer. PLOS ONE, 9(10), 2014

2. ABSTRACT

Background and aims Colorectal cancer (CRC) is one of the world's three most common cancers, and its incidence is rising. Novel biomarkers are essential for diagnostic and prognostic tools and to identify patients for targeted and individualized therapy. Covering all human cells, the carbohydrate units of glycoproteins, glycolipids, and proteoglycans are glycans. Carcinoma-related glycan structures are potential cancer biomarkers, since glycosylation evolves during carcinogenesis. Suggested to play a role in carcinogenesis are glycoproteins podocalyxin (PODXL) and regenerating islet-derived gene (REG) 4. PODXL's aberrant expression or allelic variation or both associate in different cancers with poor prognosis and unfavourable clinicopathological characteristics. Up-regulated REG4 expression occurs in inflammatory bowel diseases and also in gastrointestinal cancers. Reports on the association of REG4 expression with CRC prognosis have been mixed, however.

Material and Methods Comparison of the N-glycan profiles of 5 rectal adenomas and 18 rectal carcinomas of different stages was by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry. Tumour expression of REG4 and PODXL was evaluated by immunohistochemistry in 840 consecutive CRC patients surgically treated between 1983 and 2001. In addition we evaluated in a subgroup of 220 consecutively surgically treated CRC patients the tumour expression of MUC1, MUC2, MUC5AC, synapthophysin, chromogranin, sialyl Lewis a (sLe_a), and pauci-mannose. All patients were treated at Helsinki University Hospital (HUH).

Results Rectal adenomas and carcinomas can be distinguished from one another based on their N-glycosylation profile. Differences in N-glycosylation

existed also between carcinomas of different stages. Based on these results pauci-mannose and sLe_a were chosen for immunohistochemical analysis: in CRC sLea correlated with poor prognosis, and in advanced CRC, pauci-mannose expression correlated with poor prognosis. PODXL was an independent marker of poor prognosis in CRC. The two antibodies showed similar prognostic profiles, but their staining patterns differed, and they recognized different groups of patients with a poor prognosis. Combination of the two PODXL antibodies identified a group of patients with even worse prognosis. REG4 expression associated with MUC1, MUC2, and MUC5AC expression in CRC and was a marker of favourable prognosis in non-mucinous CRC.

Conclusion Mass spectrometry identified several carcinoma-related glycans and a method of transforming these results into immunohistochemistry was demonstrated. PODXL was a marker of poor prognosis in CRC, whereas REG4 expression predicted a favourable prognosis in non-mucinous CRC.

3. ABBREVIATIONS

ACF	Aberrant crypt foci
APCC	Australian Clinico-Pathological Staging
AJCC	American Joint Committee on Cancer
APC	Adenomatous polyposis coli
CIMP	CpG Island Methylator Phenotype
CIN	Chromosomal instability
CRC	Colorectal cancer
CRM	Circumferential Resection Margin
CT	Computed tomography
DNA	Deoxyribonucleic acid
DSS	Disease-specific survival
EGFR	Epidermal growth facro receptor
F	Deoxyhexose
FAP	Familial adenomatous polyposis
FIT	Faecal immunochemical blood test
FOBT	Faecal occult blood testing
FS	Flexible sigmoideoscopy
G	N-glycolylneuraminic acid
H	Hexose
HNPCC	Hereditary non-polyposis colorectal cancer
HUH	Helsinki University Hospital
IHC	Immunohistochemistry
kDa	Kilo dalton
KRAS	Kirsten rat sarcoma viral oncogene homologue
LHC	Left hemicolon

mAb	Monoclonal antibody
MALDI-TOF	Matrix-assisted laser desorption-ionization time-of-flight
MS	Mass spectrometry
MSI	Microsatellite instability
MRI	Magnetic resonance imaging
MUC	Mucin
N	N-acetylhexosamine
N-glycan	Asparagine-linked glycan
P	Acid ester
pAb	Polyclonal antibody
PCA	Principal component analysis
PODXL	Podocalyxin
REG4	Regenerating islet-derived gene 4
RHC	Right hemicolon
RNA	Ribonucleic acid
S	N-acetylneuraminic acid
sLe_a	Sialyl Lewis a
TIL	Tumour-infiltrating lymphocyte
TMA	Tissue microarray
TME	Total mesorectal excision
TNM	Tumour node metastasis
UICC	Union Internationale Contre le Cancer
WHO	World Health Organization

4. INTRODUCTION

Colorectal cancer is one of the world's three most common cancers, and its incidence is rising. A great majority of CRCs are sporadic, while 4 to 5% of them result from hereditary syndromes, and the estimate is that up to 20% of colorectal cancers may have some familial component. Early detection, radical surgery, and adjuvant chemotherapy are important for clinical outcome. The most crucial factor today for predicting patient outcome is stage of disease at diagnosis; roughly 40% have localised disease and another 40% regional disease. (1)

Adjuvant therapy is today standard care for Stage III patients, giving an absolute 10% increase in 5-year overall survival, but for Stage II patients, the benefit of adjuvant therapy is still unclear. In Stage II patients, T4-stage, high histological grade, vascular invasion, tumour obstruction, bowel perforation, and inadequate lymph node resection favour the need for adjuvant therapy, although only limited prospective data support this need. It would be important to identify those Stage II patients who benefit from postoperative treatment. (2)

Because many of the tumour markers studied over the years, CA19-9, CA50, CA242, and STn, represent changes in the carbohydrate structures of cancer cells, the N-glycan profiles in CRC are an interesting study subject. Glycans are the carbohydrate units of glycoproteins, glycolipids, and proteoglycans. Glycosylation evolves during carcinogenesis, meaning that carcinoma-related glycan structures are potential cancer biomarkers that can be detected by mass spectrometry (MS). To date, however, cancer-related carbohydrates have not been systematically screened by glycomic approaches, mainly due to lack of

suitable analysis technologies. The prognostic role of glycan structures can be studied by MS or they can be translated into immunohistochemical methods by finding/producing relevant antibodies.

In addition, the prognostic role of two glycoproteins in CRC was studied: podocalyxin (PODXL) and the regenerating islet-derived gene (REG) 4. PODXL is a transmembrane glycoprotein whose aberrant expression or allelic variation or both associate with poor prognosis and unfavourable clinicopathological characteristics in various cancers. REG4 belongs to a group of small secretory glycoproteins involved in cell proliferation and regeneration; it is up-regulated in inflammatory bowel diseases and also in gastrointestinal cancers. Reports on the association of REG4 expression with CRC prognosis have been mixed.

5. REVIEW OF THE LITERATURE

5.1. Epidemiology

Colorectal cancer is among the three most common cancers in both sexes, accounting for 1 in every 10 tumour types globally, with over 1.3 million new cases annually. It is the fourth most common cause of cancer death, with over 600 000 deaths. Its incidence is 1.4-fold higher in men and between different regions, the difference in incidence is 10-fold. (3)

CRC incidence has been rising in areas where it used to be low, while in Western Europe, Northern America, Australia, and New Zealand its incidence has reached a steady level or even decreased. (4). In Finland its incidence is still rising, with 2012 seeing new cases diagnosed amounting to approximately 2900. CRC is the third most common cancer in Finland with only breast cancer in women and prostate and lung cancer in men having higher incidences. Over 60% of CRC originate in the colon (5). The growing incidence of CRC in traditionally low-risk areas reflects a change in lifestyle and environmental factors, such as obesity, decreased physical activity, smoking, red-meat consumption, and high alcohol consumption.

The mortality rate from CRC in Western countries has declined, often accredited to cancer screening programs, to early detection of cancerous lesions, and to availability of improved therapies. In Finland, male mortality from CRC in 2012 was 9.9 of 100 000, making it the third most common cause of cancer death. Female mortality from CRC was 6.9 of 100 000, making it, similarly, the third most common cause. In 2012 in Finland, about 1200 individuals died from CRC. The cumulative 5-year survival of colon cancer

was 61% for female and 60% for male patients, and that of rectal cancer 65% and 62%. (5)

5.2. Etiology

Risk factors for colorectal carcinogenesis include family history of colorectal neoplasias and development of polyps. Chronic inflammation of the bowel, including ulcerative colitis and Crohn's disease, is another risk factor for CRC. Long-standing inflammation and repair is proposed to change the cellular features of the epithelium and cause DNA damage (6); 10 years of ulcerative colitis elevates the risk for CRC 10- to 30-fold. Patients with Crohn's disease are at around 1.5-fold as high a risk for CRC than is a matching population (7). Diets with high caloric intake, meat, high animal-fat intake, and especially products of pyrolysis cause increased risk for CRC, whereas diets rich in fruits, vegetables, and fibre reduce the risk (8). Alcohol consumption and smoking raise the risk for developing CRC, and smoking also the risk of CRC death (9,10).

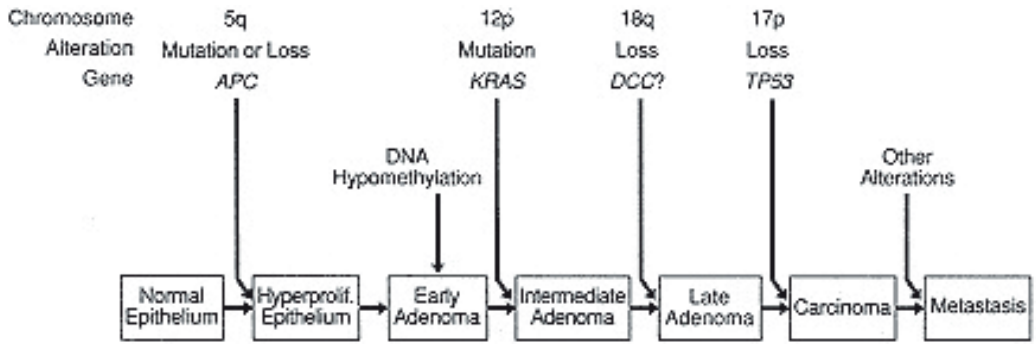
5.3. Pathogenesis

About 4 to 5% of all colorectal cancers result from hereditary syndromes, with the rest being sporadic; estimates are, however, that up to 20% of all colorectal cancers may have some familial component. A model by Fearon & Vogelstein (11) has been the paradigm of genetic alterations during colorectal carcinogenesis. This model is based on colorectal carcinogenesis in familial adenomatous polyposis; in sporadic cases the pattern and order of mutations varies more.

Aberrant crypt foci (ACF), located in the mucosal crypts, are considered to contain the earliest premalignant genetic mutations and to be precursor lesions to adenomas and carcinomas (12). The vast majority of colorectal cancers arise through adenomas, but it is hypothesized that ACF can also transform directly into cancer(11,13). The adenoma-carcinoma pathway is a slow process, a time period measured in years, even decades. Time to progression depends on the adenoma's qualities: large size, multiple adenomas, villous histology, and high-grade dysplasia considered high-risk (14). As a distinct entity among adenomas are the sessile serrated adenomas, thought to progress via a different pathway, with distinct molecular and pathological characteristics (15).

Similar to other cancers, CRC is considered to arise after accumulating mutations in tumour-suppressor genes and oncogenes, leading to dysregulation of cell homeostasis and transitions of normal cells to cancerous ones. Based on sequential analysis of the colon cancer genome, a mean of 67 genes are mutated in the genome of a CRC, and a subset of 12 key genes have been proposed as those most likely to be involved in formation of an individual carcinoma (16). Three major pathways of genetic instability are currently recognized: a chromosomal instability (CIN) pathway that accounts 70 to 85% of sporadic CRCs, a microsatellite instability (MSI) pathway that appears in 15% of sporadic CRCs, and the CpG Island Methylator Phenotype (CIMP) pathway that appears active in up to 20% of sporadic CRCs. These pathways are not always mutually exclusive, and a tumour can exhibit features from different pathways (17).

Figure 1. Colorectal cancer development according to Fearon and Vogelstein



Colorectal cancer progression involves genetic alterations in oncogenes and tumour suppressor genes Reprinted with permission from the publisher (Fearon, E. & Vogelstein, B., 1990. A Genetic Model for Colorectal Tumorigenesis. *Cell*, 61(5), pp.759–767.)

5.3.1. The chromosomal instability (CIN) pathway

Chromosomal gains or losses characterize CIN (18), resulting in aneuploidy and, subchromosomal genomic amplifications, and a high frequency of loss of heterozygosity (17). Mutations typical to CIN are: mutations in the adenomatous polyposis coli gene (*APC),* mutations in the Kirsten rat sarcoma viral oncogene homolog-gene (*KRAS)*, loss of chromosome 18q, and deletion of chromosome 17p, containing the tumour- suppressor gene *TP53* (19).

Mutations in tumour-suppressor gene *APC,* considered an early event in carcinogenesis, occur in up to 80% of sporadic adenomas and carcinomas (17,19). Inactivating mutations of the *APC* gene result in down-regulation of WNT signalling. Mutated APC protein fails to bind and degrade β-catenin, which leads to translocation of β-catenin tot the nucleus, promoting cell proliferation (20,21). This causes migration problems for proliferating and

undifferentiated cells at the bottom of colonic crypts; this is thought to lead to formation of an adenomatous polyp. Subsequent mutations are suggested to participate in carcinogenesis by affecting genes that regulate signalling pathways (22).

KRAS mutation promotes cell proliferation, transformation, and differentiation (23) and is considered an early event in carcinogenesis. It is thought to relate to tumour size. KRAS is a downstream effector of *EGFR*, which through *BRAF* activates the MAPK pathway. *KRAS* mutation is more common in large adenomas (45-60%, >2 cm) and carcinomas than in small adenomas (9%, <1 cm) (24).

Inactivation of *TP53* is considered a late event in carcinogenesis. Normally functioning p53 protein inhibits thel cell-cycle upon DNA damage, thereby providing time for DNA repair, and when irreparable genetic damage occurs, it induces pro-apoptotic genes (17).

5.3.2. The microsatellite instability (MSI) pathway

Microsatellites are short repeating sequences of DNA, ones vulnerable to errors during replication due to their repetitive manner. When functioning normally, the DNA mismatch repair system (MMR) recognises and instantly repairs these errors (25). Defective MMR presents as microsatellite instability (MSI) (26).

The majority of MSI CRSs occur sporadically due to DNA methylation of the MLH1 promoter, whereas MSI is caused by germline mutations in one of the MMR genes in the hereditary Lynch syndrome. Both sporadic and hereditary

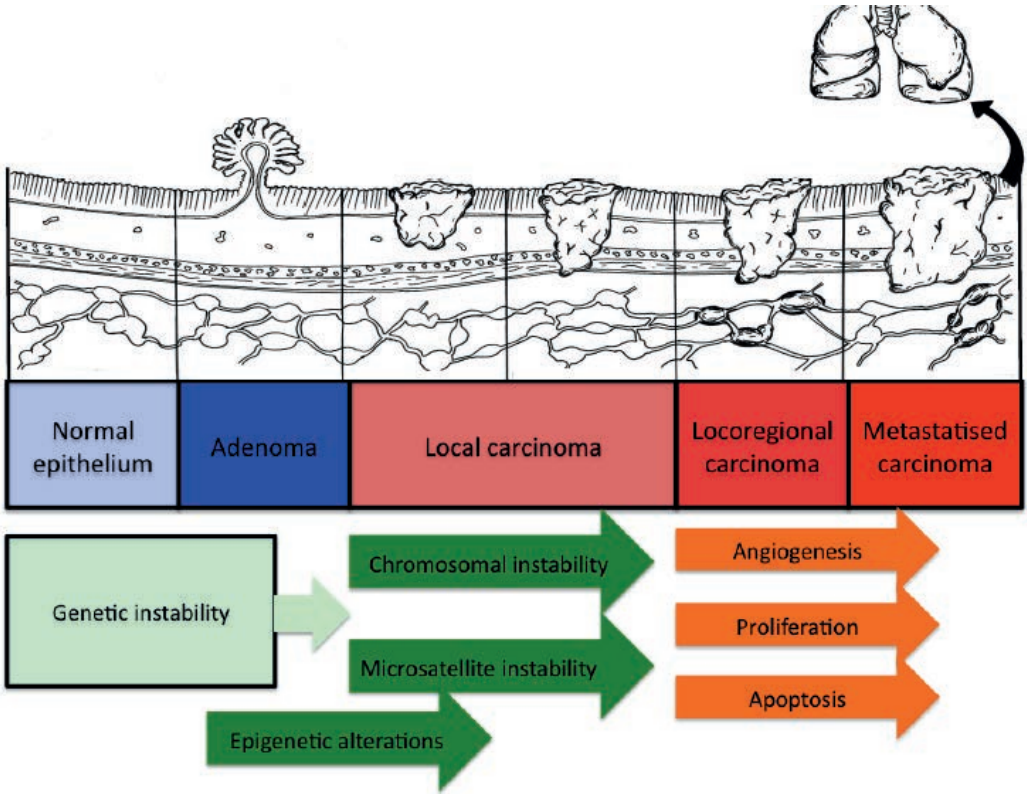
MSI tumours have similar biology, but the precursor lesion in Lynch syndrome is a traditional adenoma, whereas the predominant precursor lesion in sporadic MSI is an SSA. (19). MSI tumours are associated with location in the proximal colon and are often poorly differentiated (27). Sporadic MSI tumours are also more common in women and in elderly patients (26).

5.3.3. The CpG Island Methylator Phenotype pathway

Changes in gene expression or function caused by amechanism not affecting DNA sequence, such as DNA methylation or histone modification, are called epigenetic alterations (25).

Short sequences rich in CpG dinucleotide, commonly found the in promoter region of genes, are prone to DNA methylation. Methylation of these regions causes loss of gene expression, and typical examples in CRC are *APC* and *MLH1*. Methylation silencing the MLH1 expression thus causes sproradic MSI (19,25,27). Hypermethylation, evident in up to 20% of CRC, is called the CpG Island Methylator phenotype (CIMP). CIMP is associated with proximal location, old age, female sex, mucinous and poor differentiation, MSI, and BRAF mutation (26,28)

Figure 2. Model of colorectal carcinogenesis



Role of different pathways in the transformation of normal mucosa to metastatic cancer. Adapted from publication III, permission by CC BY.

5.4. Common hereditary syndromes

The most common of hereditary syndromes, comprising 2 to 3% of all colorectal cancers, is Lynch syndrome (or hereditary non-polyposis colorectal cancer, HNPCC). It is an autosomal dominant disease with a high lifetime risk for colorectal cancer (29,30) caused by mutations in DNA MMR genes, most commonly in the *MLHI* and *MSH2* genes (31). Patients are typically younger (40-50 years) than in sporadic SCR and metachronous or synchronous cancers occur in one-fifth of them (32). Prognosis for colorectal cancers caused by a

mutated MMR gene is better than that of sporadic colorectal cancer (33). Because of the high lifetime risk for colorectal cancer, the recommendation is regular screening by colonoscopy (34).

The second most common hereditary syndrome, causing 1% of all colorectal cancers, is familial adenomatous polyposis (FAP). This results from mutations in the *APC* gene, resulting in hundreds to thousands of adenomatous polyps in the colorectum. The lifetime risk for colorectal cancer is virtually 100% at the median age of 40 (35).

5.5. Screening

As transition from detectable adenoma to carcinoma may take at least a decade, and the transition of early invasive cancer to overt disease several years, CRC is a good candidate for cancer screening. Faecal occult blood testing (FOBT) is the most common test to screen for CRC and the only one recommended by the EU (36). FOBTs need to be done repeatedly to enhance sensitivity, either each or every other year, since CRCs bleed intermittently (37). Screening by FOBTs reduces relative risk for CRC mortality by 16% (38). Two tests are available: guaiac-based FOBT (gFOBT) and the faecal immunochemical blood test (FIT). FIT has a higher sensitivity but a lower specificity for late adenomas and carcinomas than does gFOBT (39). Flexible sigmoideoscopy (FS), allows inspection and the extraction of tissue specimens from the distal colon, has reduced relative CRC mortality by 18 to 23% (40,41). No such effect emerged, however in one population-based study (42). Colonoscopy allows inspection and tissue sampling of the whole colon with a sensitivity and specificity of practically 100%, suggesting it to be the perfect screening tool. Colonoscopy

has reduced relative mortality from CRC by 40 to 60% (43). However, its high cost, invasiveness and possible painfulness reduce its screening utility.

5.6. Diagnosis

5.6.1. Symptoms

Among, common symptoms for CRC are altered bowel habits, anaemia, haemotochezia, but many patients lack these symptoms altogether. Signs of advanced disease are fatigue, weight loss, abdominal pain, and bowel obstruction or perforation. Liver enlargement and ascites are symptoms of metastatic disease. (44)

5.6.2. Diagnosis

Clinical rectal examination can detect over half of all rectal tumours (45), but only large colonic tumours are palpable.

In standard practise diagnosis is based on endoscopy, preferably total colonoscopy. This allows precise localisation and biopsy of the lesion, detection of possible synchronous cancerous or precancerous lesions, and removal of polyps. If a complete preoperative colonoscopy is for some reason impossible, then virtual colonoscopy or CT colonography are possible substitute options. If not carried out preoperatively, then a complete colonoscopy is recommended 3 to 6 months after the operation. In rectal cancer, a rigid rectoscopy may enable evaluation of the position of the lowest part of the tumour relative to the anal canal, allowing the choice of an appropriate surgical technique.

5.6.3. Preoperative staging

Preoperative clinical examination, instrumental screening, and laboratory tests can detect or exclude metastatic disease. Liver enzymes are usually screened preoperatively, although they can be normal even in the presence of liver metastases. Computed tomography (CT) of the abdomen is performed to allow detection of metastatic spread to the liver, and it also helps in the evaluation of tumour size, location, relationship to adjacent organs, and invasion depth. The sensitivity of CT for lymph node metastasis is 76% and specificity 55%, and for detection of distant metastases 85% and 98%. Chest CT is the first choice in a search for lung metastases (46). Routine use of positron emission tomography (PET) is not recommended at the time of diagnosis, because in the great majority of patients it does not modify the treatment approach (47).

In addition to chest and abdomen CT, magnetic resonance imaging (MRI) serves for local preoperative staging in rectal cancer. MRI allows evaluation of depth of invasion, enlargement of lymph nodes, and distance of the tumour from the anal canal, all of which affect the choice of preoperative treatment and surgical technique.

5.7. Management

To improve patient prognosis, CRC treatment should involve multidisciplinary teamwork (48).

5.7.1. Surgical and preoperative treatment

5.7.1.1. Colon cancer

Tumour location determines the choice of operative technique: tumours from the caecum to the ascending colon are operated on by right hemicolectomy, tumours from the hepatic flexure to the right side of tranverse colon by extended right hemicolectomy, tumours from the left side of the tranverse colon to the splenic flexure by extended left hemicolectomy, and tumours from the descending colon to the sigmoid by left hemicolectomy. A proximal and distal margin of at least 5 cm is recommended, and en-bloc excision of the mesocolon with proximal ligation of the vessels improves radicality in locally advanced disease and improves staging. Invasion of the tumour into neighbouring organs requires en-bloc removal with healthy-tissue margins. On some occasions, local and small tumours can be removed by endoscopic resection (49).

Laparoscopic surgery for colon cancer has several short-term advantages over open laparotomy, such as smaller incisions, lower usage of analgetics, reduced operative trauma leading to faster restoration of gastrointestinal motility, and a faster return to full activity and work (50). In laparoscopically operated patients surgical morbidity and post-operative complications are lower (51). Oncological outcomes between laparoscopic and open surgery do not differ. (52)

At HUH, all patients with suspected stage III to IV disease at diagnosis are discussed at a multidisciplinary colon cancer meeting including a GI surgeon, a liver surgeon, an oncologist, a radiologist, and a pathologist. The treatment plan decided upon is then explained to the patient.

5.7.1.2. Rectal cancer

The aim of rectal cancer surgery is to minimize risk for residual disease in the pelvis that often causes disabling local recurrence, and at the same time to minimize possible acute and late morbidity. What is also important is as often as possible, to retain sphincter function (53). Total mesorectal excision (TME) is the gold standard for a rectal cancer operation: combined with a circumferential resection margin (CRM), it results in low recurrence rates and a good oncological outcome. (54)

Laparoscopic surgery for rectal cancer has short-term benefits similar to those of open surgery, just as it has in colon cancer (4,50). Oncological result are similar between laparoscopic and open surgery.(11,55,56)

To reduce local recurrences, preoperative radiotherapy of 25GY in 5-GY fractions over 5 days followed by immediate surgery (TME) is recommended for most T3 and some T4 tumours (e.g. with only peritoneal involvement) and for tumours with lymph-node involvement. Here, an option for short radiotherapy is a longer 50GY 1.8 GY/fraction with or without 5-FU. Preoperative radiotherapy is more effective and less toxic than is postoperative radiotherapy.

For most locally advanced tumours: T3 with lymph-node growth to the mesorectal fascia and T4 with non-readily resectable growth to neighbouring organs, a longer radiotherapy of 50GY in 1.8-GY fractions with 5-FU-based chemotherapy is recommended, followed by surgery 6 to 8 weeks later. (50,57)

At HUH all rectal-cancer patients' care is centralized in a rectal-cancer care unit. Each patient's preoperative radiological images and histological samples are discussed at a multidisciplinary rectal cancer meeting including a GI surgeon, a liver surgeon, an oncologist, a radiologist, and a pathologist. A treatment plan is then decided upon and then explained to the patient.

5.7.2. Adjuvant treatment

Adjuvant therapy aims to improve prognosis and reduce risk for recurrence, and in CRC it is routine for stage-III patients, in whom it provides a 10% absolute increase in 5-year survival. (2,51). For stage II, it is not the routine recommendation for unselected patients, since the benefit in overall survival is quite small. (2,52) Adjuvant therapy is only recommended for high-risk stage-II patients: T4 stage, lymph nodes sampling <12, poorly differentiated tumour, bowel obstruction or tumour perforation, and tumour with vascular or lymphatic or perineural invasion. A doublet schedule with oxaliplatin and a fluoropyrimidine is standard. (53,58)

In rectal cancer, adjuvant therapy can be given to stage-III and high-risk stage-II patients, even though scientific evidence of benefit is lower than in colon cancer. (54,57)

5.7.3. Treatment of metastatic colorectal cancer

Liver, lung, peritoneum, and other colonic segments are the most common sites for metastasis that can receive curative surgery based on metastasis size and location. At the time of their diagnosis, 10 to 20% of liver metastases can be

resected and of unresectable tumours 10 to 15% can be resected after oncological treatment (59). No difference exist in perioperative or long-term outcomes between simultaneous or staged resection of liver metastases and primary tumour (60). Lung metastases are rare at the time of diagnosis and only less than 5% of them can be curatively operated on, most often due to other metastases (61).

At HUH, a patient with metastatic CRC with readily resectable metastases usually receives adjuvant therapy after surgery for 6 months as combination chemotherapy. If the metastases are not readily resectable, combination therapy continues for 3 months before possible surgery, and for at least another 3 months after surgery.

5.7.4. Palliative surgery

In some patients, because of too-advanced local or systemic disease or poor overall condition, no surgical resection of the primary tumour can be recommended. Then an obstructive tumour can be treated by stenting the tumour, or by a decompressing stoma or with by-pass surgery.

5.8. Prognosis

During recent decades, prognosis of patients with colorectal cancer in many countries has improved. In high-income countries, 5-year relative survival is almost 65%, while in low-income countries remaining below 50%. Survival is higher for younger patients, and is higher for women than for men in younger patients. (62) The age-standardised 5-year colon-cancer-specific relative survival ratio in 2007-2009 in Finland was 60% for men and 61% for women,

with corresponding figures of 62% and 65% for cancers of the rectum, rectosigmoid, and anus. (5).

5.8.1. Clinocopathological prognostic factors

5.8.1.1. Stage

The most important prognostic factor is stage at diagnosis (63). The tumour node metastasis (TNM) staging system is now the one most commonly used, published first by the Union Internationale Contre le Cancer (UICC) in 1949 (64). Later it was integrated into the staging system of The American Joint Committee on Cancer (AJCC), creating the now-used TNM classification, with its latest and 7th edition published in 2010. Another staging system is the older Dukes classification, first published in 1932 (65) for rectal cancer only, adapted for the colon and rectum by Astler and Coller in 1953 (66), modified by Turnbull in 1967 to include unresectable tumours and distant metastases (67), and again by Australian Clinico-Pathological Staging (ACPS) in 1982 (68). In Dukes stage A, the tumour invades the submucosa or at most the muscularis propria, and in a stage B tumour invades into or through the bowel wall into the surrounding fat. In stage C there is local lymph node metastasis, and in stage D distant metastasis or local residual tumour after surgery.

In the TNM 7th classification of colorectal cancer, T represents tumour infiltration, N represents lymph-node involvement, and M stands for distant metastasis (Table 1). The original TNM stage is based on preoperative information, but for planning surgical and oncological treatment, the choice is pTNM, and it also includes data from the pathology report.

The pathology report should include a definition of tumour site and size, presence of tumour perforation, histological type and grade, extent of tumour invasion (T stage), resection margins, tumour deposits, lymphovascular or neural invasion, tumour budding, number of lymph nodes studied and possible lymph-node metastases (N stage), and the possible involvement of other organs (M stage). (46)

Table 1. TNM stages by UICC, 7th edition, 2010

T – Primary Tumour

TX Primary tumour cannot be assessed
T0 No evidence of primary tumour
Tis Carcinoma in situ: intraepithelial or invasion of lamina propria
T1 Tumour invades submucosa
T2 Tumour invades muscularis propria
T3 Tumour invades through muscularis propria into subserosa or into non-peritonealized pericolic or perirectal tissues
T4a Tumour perforates visceral peritoneum
T4b Tumour directly invades other organs or structures

N – Regional Lymph Nodes

NX Regional lymph nodes cannot be assessed
N0 No regional lymph node metastasis
N1 Metastasis in 1 to 3 regional lymph nodes
N1a Metastasis in one regional lymph node
N1b Metastasis in 2–3 regional lymph nodes
N1c Tumour deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis
N2 Metastasis in 4 or more regional lymph nodes
N2a Metastasis in 4–6 regional lymph nodes
N2b Metastasis in 7 or more regional lymph nodes

M – Distant Metastasis

MX Distant metastasis cannot be assessed
M0 No distant metastasis
M1 Distant metastasis
M1a Metastasis confined to one organ or site (for example, liver, lung, ovary, nonregional node)
M1b Metastases in more than one organ/site or the peritoneum

Table 2. Survival according to modified Dukes´ and TNM stage

Dukes[+]	Stage	T	N	M	5-year survival[*]
-	0	Tis	N0	M0	
Dukes´A	I	T1-T2	N0	M0	92.5
Dukes´B	IIA	T3	N0	M0	83.6
Dukes´B	IIB	T4a	N0	M0	76.3
Dukes´B	IIC	T4b	N0	M0	58.8
Dukes´C	IIIA	T1-2	N1/N1c	M0	83.1
		T1	N2a	M0	
Dukes´C	IIIB	T3-T4a	N1/N1c	M0	63.8
		T2-T3	N2a	M0	
		T1-T2	N2b	M0	
Dukes´C	IIIC	T4a	N2a	M0	35.2
		T3-T4a	N2b	M0	
		T4b	N1-2	M0	
DukesD	IVA	any T	any N	M1a	10.4
Dukes´D	IVB	any T	any N	M1b	

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[+]Additional information to the original table *data for 5-year disease-specific survival from (69)

5.8.1.2. Grade

Histological grade is based on glandular formation of the tumour. Histological grade is divided into four groups according to the WHO classification: well differentiated (1), moderately differentiated (2), poorly differentiated (3), and undifferentiated (4). Low grade predicts better prognosis (15).

5.8.1.3. Histological type

Over 90% of cololorectal carcinomas are adenocarcinomas, originating from epithelial cells of the colorectal mucosa (15). Mucinous adenocarcinoma is a subtype of adenocarcinoma, in which over 50% of the tumour consists of extracellular mucin. The prognostic role of mucinous differentiation compared to that of conventional adenocarcinoma varies between studies (70,71) Rare

types of colorectal carcinomas include signet-ring cell, medullary, adenosquamous, neuroendocrine, undifferentiated, and small cell carcinomas (15).

5.8.1.4. Tumour deposits

Focal aggregates of adenocarcinoma located in the pericolic or perirectal fat, discontinuous with the primary tumour, are called tumour deposits. Their origin is heterogenous, and they represent venous invasions, lymphatic invasion, nerve-sheath invasion, and continuous growth. (72,73). They are more common in T3N+-disease than in local disease and indicate poor prognosis (74).

5.8.1.5. Lymphatic, vascular, and perineural invasion

Invasion of the tumour into extramural veins means increased risk for hepatic metastasis (75) and vascular invasion is a marker of poor prognosis (76). Tumour invasion into lymphatic vessels indicates poor prognosis (77), as does perineural invasion as well (78).

5.8.1.6. Tumour immunity

A high density of tumour-infiltrating lymphocytes (TILs) is associated with good prognosis, and low levels with poor prognosis (79) TILs are associated with the MSI-H phenotype, thought to result from the existence of frameshift antigens leading to their recognition by the immune system as tumour antigens (80).

5.8.1.7. Tumour budding

Tumour budding is defined as cluster of undifferentiated cells detaching from the surrounding tumour at the CRC invasive edge (81). It is thought to represent epithelial to mesenchymal transition, to represent an early event in invasion and metastasis, and to be a factor indicating poor prognosis (82).

5.8.1.8. Surgical margins

In rectal cancer when TME is performed, in addition to proximal and distal margins of resection, the margin around the mesorectum (CRM) is highly important. A positive CRM means elevated local recurrence rates and poorer survival when present in the rectal margins (83,84). In rectal cancer, CRM between 1 and 2 mm and for colorectal cancer distal margin of 2 cm, and proximal margin of 5 cm are considered adequate (85).

5.9.1.9. Emergency surgery

Between 15 and 30% of CRCs present as a surgical emergency, with most caused by obstruction (78%) or perforation (10%) (86,87). Patients who undergo emergency surgery tend to more often have metastases and have a higher T- and N-stage, also with vascular and perineural invasion often seen (88).Their overall survival is poorer for those patients needing emergency surgery than for patients with elective surgery (89,90).

5.8.1.10. MSI

Analysis of microsatellite instability (MSI) allows identification of patients with hereditary CRC, but it also has prognostic and predictive implications. The MSI status of CRC patients is usually stratified into two classes based on the

number of unstable loci: MSI high (MSI-H) and MSI low/MSI stable (MSI-L)/MSS). MSI-H patients have a better overall survival (91) and MSI status also may prove useful as a predictive marker as well (62).

5.8.1.11. Inflammatory colorectal cancer

In addition to tumour characteristics also host-response factors determine prognosis (92). The host's immune system plays a prognostic role in various cancers. These responses can be examined by the circulating concentrations of acute-phase proteins. A systemic inflammatory response has been a sign of poor prognosis in CRC (93,94) and in other cancers (95). Glascow Prognostic Score, comprising albumin and C-reactive protein, is an independent marker of poor prognosis in CRC (96).

5.9. Glycans

Glycans, the carbohydrate units of glycoproteins, glycolipids, and proteoglycans, cover all human cells. Six main families of glycans exist: (1) asparagine-linked (N-linked) glycans of many glycoproteins, (2) serine-or threonine-linked (O-linked) glycans predominating on glycoproteins and membrane-bound mucins, (3) the glycosaminoglycans that present as free polysaccharides or as part of proteoglycans, (4) the glycosphingolipids consisting of oligocosaccharides binding to ceramide, (5) the glycosylphosphatidylinositol (GPI)-linked proteins (proteins bearing a glycan linked to phosphatidylinositol, and (6) various cytoplasmic and nuclear proteins containing *O*-linked *N*-acetylglucosamine (O-GlcNAc) (97)

Around 1% of the human genome participates in the biosynthesis of glycans (98-100).

This biosynthesis is the most complex post-translational modification of proteins, and the great variability in glycan structures means a tremendous ability to fine-tune the glycoproteins' chemical and biological properties. This glycosylation process occurs most abundantly in the Golgi apparatus and the endoplasmic reticulum, but also occurs in the cytoplasm and the nucleus (101,102). A majority of glycoconjugates are localized to cell surfaces, where glycans participate in events essential for cell viability and function, such as cell adhesion, motility, and intracellular signalling (102,103). Changes in these functions are essential steps seen when cells transform into malignant ones, and these are also reflected in changes of a cell's glycan profile, observed in many cancers (104-106). Specific structural changes in glycans may serve as cancer biomarkers (104,107-109), and changes in glycosylation profiles are related to tumours cells' aggressive behavior(110-112). The incorporation of foreign monosaccharides from the environment has been proposed to promote carcinogenesis through systemic inflammation, as is the case of incorporation of nonhuman sialic acid N-glycolylneuraminic acid from red meat to human cells (113).

5.9.1. N-glycans

Cancer-associated N-glycan structures may play specific roles in supporting tumour progression: growth (114,115), invasion (116,117), and angiogenesis (118). Changes in the N-glycan profile emerge in various cancers, including lung (119,120), breast (121), and colorectal cancer (120,122). Moreover, serum

N-glycosylation profiles from patients with CRC and those of healthy controls differ. (123).

5.10. Biomarkers

5.10.1. Serum markers

Carcinoembryonic antigen (CEA) is a serum glycoprotein that participates in carcinoma cell adhesion, promoting tumour-cell aggregation, and possibly promoting metastasis (124). CEA is overexpressed by several adenocarcinomas: colorectal, breast and lung (125). Smokers have higher serum-CEA levels than do non-smokers, with elevation of serum CEA levels seen in various acute and chronic inflammations (126) and in cholestasis (127). Elevated CEA levels occur in 20% of CRCs (128). Preoperative CEA has some prognostic value (129), but in the clinic it is used for follow-up. Increasing CEA levels in follow-up predict recurrence/metastasis, and including CEA measurements in follow-up improves prognosis (130,131).

5.11. Tissue biomarkers in this thesis

5.11.1. Podocalyxin

The discovery of podocalyxin-like 1 (PODXL) was originally in kidney podocytes (132), but it is also expressed by vascular (133) and breast epithelium (134), and haematopoietic progenitors (135). It is an anti-adhesive type I transmembrane glycoprotein able to undergo extensive sialylazation and O-glycosylation. Estimated peptide mass for PODXL is 59kDa, and

prostranslational processing yields a mature glycoprotein of 165kDa (136). PODXL is recognised as a stem cell marker (137) and is closely related to CD34 and endoglycan. Through its connections to intracellular proteins and to extracellular ligands, it participates in regulation of cell morphology and adhesion (138-141). The role of PODXL in cancer is not thoroughly understood, but it is thought to participate in epithelial-mesenchymal transition (142), and interact with various mediators of metastasis (139-141,143,144).

In various cancers, such as renal cell carcinoma, breast, colorectal, urothelial bladder, testicular, and pancreatic cancer, PODXL has been reported to be expressed aberrantly, and in the first four also has been an independent marker of poor prognosis (134,139,145-148). Membranous PODXL expression has been suggested to correlate, in CRC and urothelial bladder cancer, with poor prognosis (146,147,149). Association of germline variants of PODXL with development of prostate cancer and also with a more aggressive form has been reported (143). Occurrence of missense mutations in PODXL elevates the risk for prostate cancer development of cancer by 50% and an in-frame deletion associates with more aggressive tumours (143).

5.11.2. REG4

Regenerating islet-derived gene (REG) proteins represent a group of small secretory proteins involved in cell proliferation and regeneration, and also participating in formation of the immune system (98,100) . They belong to the calcium-dependent lectin (C-lectin) superfamily and based on their primary structure are divided into four families, REG I to IV. Distinctive of the other

REG proteins, REG4 binds polysaccharides independently of calcium (101). REG I to III genes are located on chromosome 2p12, while that of REG4 is on 1p12-13. REG4 was first cloned and identified by Hartupee et al (103) and by Kämäräinen et al (104). REG4 contains a 158 amino acids, at a weight of 18kDa, and it is physiologically expressed in the colon and the small intestine, with high expression in enteroendocrine cells (104,107). In the gastrointestinal epithelium, REG4 is activated during specific phases of differentiation and maturation, its expression is spatially specific, and it has been suggested to support mucinous and neuroendocrine differentiation (98,100,150,151).

Up-regulated REG4 expression occurs in inflammatory bowel diseases (IBD) (101,104) and also occurs in many malignancies: colorectal, gastric, and pancreatic cancers (103,152-154). REG4 participates in carcinogenesis and tissue regeneration, acts as an antiapoptotic factor, and promotes proliferation and invasion (104,155). Much still remains elusive as to REG4´s ultimate physiological and pathological roles. Expression of REG4 has predictive and prognostic value in many GI-tract cancers (104,107,152,153,156,157). The findings in CRC have been mixed: increased expression is, according to Numata et al (158), a sign of poor prognosis, whereas in other studies no association with prognosis has emerged (159,160).

5.11.3. Mucins

Mucins are high-molecular weight glycoproteins, defined by tandem repeat sequences with highly O-glycosylated serine and threonine residues (161). They are widely expressed by epithelial cells and are classified into membrane-associated and secretory glycoproteins. Of the mucins studied here, MUC4

(mucin 4) and MUC5AC belong to secreted glycoproteins, while MUC1 and MUC2 are transmembranous (162).

5.11.3.1. MUC1

The *MUC1* gene location is in chromosome 1q21, and MUC1 is expressed on the apical surfaces of secretory epithelial cells. During carcinogenesis, MUC1 overexpression, aberrant glycosylation, and cytoplasmic localisation may play a role in anchorage-independent growth and resistance to apoptosis (162-164). In CRC, MUC1 expression is associated with mucinous phenotype, increased proliferation rate, invasiveness, metastatic disease, and poor prognosis (165,166).

5.11.3.2. MUC2

MUC2 is found predominantly in colorectal goblet cells; its expression has been proposed to decrease during CRC progression and to act as a marker of poor prognosis (167), but not all reports support this (168).

5.11.3.3. MUC4

MUC4, physiologically expressed in the epithelium of the respiratory, gastrointestinal, and genital tracts (169), is frequently overexpressed in CRC, where it associates with poor prognosis (170), but the mechanism regulating its expression in CRC remains elusive.

5.11.3.4. MUC5AC

MUC5AC is mainly expressed in the tracheo-bronchial and gastric mucosa. In CRC its absence is associated with lower differentiation and poor prognosis. (171,172)

5.11.4. Sialyl Lewis a

Sialyl Lewis a (sLe_a), known in the clinic as CA19-9, is a sialylated glycoconjugate found on the terminal chains of glycolipids and N-/O-glycoproteins (173,174) Sialylation is a common phenomenon in glycoconjugates of malignant cells, and overexpression of sLe_a is also seen in various carcinomas (175). As the ligand of E-and P-selectin (176), sLe_a overexpression facilitates tumour angiogenesis and haemotogenous metastasis (177). This sLe_a overexpression appears concomitantly with loss of mucosal homeostasis and creation of tumour progression facilitating immune status (178,179). In CRC overexpression of sLe_a associates with neoplastic transformation and poor prognosis (178,180). The sLe_a antigens are released into the bloodstream; high serum levels of sLe_a associate with poor prognosis in CRC and other GI tumours (181). In clinical practise CA19-9, serves for diagnosis and follow-up of various malignancies, especially for pancreatic cancer, but for also CRC (129).

5.11.5. Paucimannose

Paucimannosidic N-glycan stuctures are rare in vertebrates. Paucimannose is a product of lysosomal exoglycosidases, whose high levels appear in CRC tissue as well as in serum (182,183). Paucimannose is up-regulated in CRC compared

to levels in healthy tissue (122), but its role in carcinogenesis is still very much unclear.

5.11.6. Markers of neuroendocrine differentiation

Poorly differentiated colorectal adenocarcinoma often retains the capacity for neuroendocrine differentiation (NED) (184). NED in CRC ranges from 12 to 78%, most likely due to differing diagnostic markers and standards. In CRC NED is a marker of poor prognosis (185,186). Several biomarkers serve to diagnose NED, with different sensitivities and specificities; those most widely used are choromogranin a and synaptophysin.

6. AIMS OF THE STUDY

- To discover the differences in N-glycosylation between rectal adenomas and carcinomas, and within carcinomas of different stages
- To translate these glycomic result into prognostic evaluation by immunohistochemistry
- To discover the association of PODXL with clinicopathological parameters and its role as an prognostic marker in CRC by two different antibodies
- To discover the association of REG4 with clinicopathological parameters and other intestinal markers, and to evaluate its role as a prognostic marker in CRC

7. PATIENTS AND METHODS

7.1. Patients (I-IV)

All patients in the studies had undergone surgery at the Department of Surgery, HUH, and tissue samples were stored in the archives of the HUH Department of Pathology.

The study population for immunohistochemistry comprised 840 consecutive colorectal cancer patients undergoing surgery in 1983-2001. (I-II, IV) A subgroup of 240 comprised consecutively operated patients from between 1998 and 2001 (III-IV). PODXL and REG4 were studied in the whole population; MUC1, MUC2, MUC4, MUC5AC, CA19-9, and paucimannose in the subgroup. The Finnish Population Register Centre provided the follow-up vital-status data needed to compute survival statistics, and Statistics Finland provided cause of death for all those deceased. Median age at diagnosis was 66, with a median follow-up of 5.1 years (range 0-25.8). The 5-year disease-specific survival rate was 58.9% (95%Cl 55.0-62.8%). For the subgroup, median age at diagnosis was 67 with a median follow-up of 6.0 years (range 0-13.2). The 5-year disease-specific survival rate was 64.8% (95%Cl 58.1-71.5%). Table 3.

Table 3. Clinicopathological characteristics of the study population for immunohistochemistry

	Study population 1983-2001	Subgroup of 1998-2001
n (%)	**840**	**220**
Age. years		
<65	360 (42.9)	101 (45.9)
≥65	480 (57.1)	119 (54.1)
Gender		
Male	466 (55.5)	134 (60.9)
Female	374 (45.5)	86 (49.1)
Dukes		
A	125 (14.9)	34 (15.5)
B	294 (35.0)	71 (32.3)
C	231 (27.5)	70 (31.8)
D	190 (22.6)	45 (20.5)
Grade (WHO)		
1	29 (3.5)	8 (3.7)
2	571 (68.4)	165 (76.7)
3	202 (24.2)	37 (17.2)
4	33 (4.0)	5 (2.3)
Missing	5	5
Side		
Right	227 (27.0)	50 (22.7)
Left	613 (73.0)	170 (77.3)
Location		
Colon	429 (51.1)	87 (39.5)
Rectum	411 (48.9)	133 (60.5)
Histology		
Adenomatous	749 (89.3)	208 (95.0)
Mucinous	90 (10.7)	11 (5.0)
Missing	1	1

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For MS (III) analysis we chose 18 rectal carcinoma patients (4 each at stages I-III and 6 at stage IV) and 5 rectal adenoma patients. All selected cases were blood group A Rh+ in order to minimize the possible influence of blood group antigens on glycan profiles. Detailed patient characteristics are in Table 4.

Table 4. Description of the sample cohort for mass spectrometry

Stage[a]	TNM[b]	Sex	Age[c]	Tumour size(cm)	Tumour histology
Adenoma		F	84	Large	Severe dysplasia
Adenoma		F	79	Large	Severe dysplasia
Adenoma		F	72	2	Severe dysplasia
Adenoma		M	64	10	Severe dysplasia
Adenoma		M	52	2	Severe dysplasia
A	T2N0M0	F	49	4	Adeno, G1
A	T2N0M0	M	59	3	Adeno, G2
A	T2N0M0	M	59	14	Adeno, G2
A	T2N0M0	M	53	2	Adeno, G2
B	T3N0M0	M	87	5	Adeno, G2
B	T3N0M0	M	71	7	Adeno, G2
B	T3N0M0	F	76	7	Adeno, G2
B	T3N0M0	M	56	5	Adeno, G2
C	T3N1M0	M	74	3	Adeno, G2
C	T3N1M0	M	61	4	Adeno, G2
C	T3N1M0	F	55	5	Adeno, G2
C	T3N2M0	M	84	4	Adeno, G2
D	T3N1M1	F	56	7	Adeno, G2
D	T3N2M1	M	82	5	Adeno, G2
D	T3N2M1	M	66	5	Adeno, G3
D	T4N2M1	M	28	6	Adeno, G3
D	T3N1M1	M	50	5	Adeno, G3
D	T3N2M1	F	64	3	Adeno, G3

[a]Dukes A-D, [b]TNM, tumor node metastasis, [c]Age at diagnosis, Adeno=Adenocarcinoma, G=Grade(1-4, WHO). Adapted from Study III, permission by CC BY.

7.2. Tumour tissue specimens (I-IV)

Formalin-fixed and paraffin-embedded tumour samples were obtained from the archives of the Department of Pathology, HUH. An experienced pathologist marked representative areas of tumour samples on haematoxylin- and eosin-stained tumour slides. Three 1.0-mm-diameter punches from each sample were mounted on recipient paraffin blocks with a semiautomatic tissue microarray instrument (Beecher Instruments, Silver Spring, MD,USA) as described (187)

7.3. Production of monoclonal PODXL antibody HES9 (I)

For the novel monoclonal antibody (mAb) HES9 used in Study I, immunization of mice was with the undifferentiated human embryonic (hES) stem cell line SA167 (Cellartis, Göteborg, Sweden, www.cellartis.com). By conventional hybridoma technology, hybridoma cell lines were established to produce mAbs against hES cells. Mimotope analysis, immunoprecipitation, and mass-spectrometry identified the target antigen as podocalyxin. For a detailed description see Supplementary file 1 of Study I.

7.4. Immunohistochemistry (I-IV)

First, the tumour tissue microarray blocks were freshly cut into 4-µm sections, fixed on slides, and dried at 37°C for 12 to 24 hours. After deparaffinization in xylene and rehydration through a gradually decreasing concentration of ethanol to distilled water, slides were treated in a PreTreatment module (Lab Vision Corp., Fremont, CA, USA) in antibody-specific buffer for 20 min at 98°C for antigen retrieval. Staining of sections was performed in an Autostainer 480 (Lab Vision) by the Dako REAL EnVision Detection system,

Peroxidase/DAB+, Rabbit/Mouse (Dako, Glostrup, Denmark). Tissues were incubated with the chosen antibody for one hour at room temperature. Antibodies and variations in pre-treatment, dilution, and positive control are in Table 5.

Table 5. Antibodies for immunohistochemistry

Antibody	Clone	Company	Pre-treatment	Dilution	Positive control
PODXL HES9	mAb	In-house	Tris-HCl (pH 8.5)	1:500	Kidney
PODXL HPA002110	pAb	Atlas Antibodies	Tris-HCl (pH 8.5)	1:250	Kidney
CA19-9/sLe$_a$	mAb,NCL-L-CA19-9	Novocastra, UK	Tris-HCl (pH 8.5)	1:300	Colon
Paucimannose	mAb	a	Tris-HCl (pH 8.5)	1:100	Colon
REG4	mAb	b	Tris-HCl (pH 8.5)	1:50	Colon
MUC1	mAb, Ma552	Novocastra, UK	Citrate (pH 6.0)	1:25	Stomach
MUC2	mAb, Ccp58	Novocastra, UK	Citrate (pH 6.0)	1:100	Colon
MUC4	mAb, 1G8	Invitrogen,USA	Tris-EDTA(pH 9.0)	1:100	Colon
MU5AC	mAb, CLH2	Novocastra, UK	Citrate (pH 6)	1:50	Stomach
Synaptophysin	mAb 27G12	Novocastra, UK	Tris-EDTA(pH 9.0)	1:200	Colon
Chromogranin	mAb, 5H7	Novocastra, UK	Tris-EDTA(pH 9.0)	1:2000	Colon

[a]Pauci-mannose antibody (188) mAb= monoclonal antibody [b] REG4 antibody (189). Antibody host: mouse

7.5. Scoring of samples (I-IV)

Tumour specimens were scored independently by two researchers who were blinded to clinical data and outcome. Differences in scoring were discussed until consensus. With all antibodies, the highest score of the triplicates of each sample was considered representative for analysis.

In Study I, PODXL expression by mAb HES9 was mainly cytoplasmic, with membranous positivity only seen with strong cytoplasmic staining. It was scored as negative cytoplasmic staining as 0, weakly positive as 1, moderately positive as 2, and strongly positive as 3.

In Study II, PODXL expression by pAb HPA002110 was cytoplasmic in tumour cells, but in some cases a distinct membranous expression was visible, which did not always correlate with intensity of cytoplasmic expression. Expression scoring: cytoplasmic staining as 0-2 (negative-moderate-strong) and in case of distinct membranous staining as 3, regardless of the intensity of the cytoplasmic staining.

In Study III, sLe_a and paucimannose expression were cytoplasmic and scored as negative-low-moderate-high (0-3)

In Study IV, REG4, MUC1, MUC2, MUC4, MUC5AC, synaptophysin, and chromogranin expressions were cytoplasmic and scored as follows: REG4 cytoplasmic expression was scored in tumour cells as either negative or positive. MUC1 and MUC2 expressions were cytoplasmic in tumour cells and were scored as negative-low-moderate-high (0-3) according to intensity. MUC4, MUC5AC, synapthopysin, and chromogranin cytoplasmic expressions were scored as either negative or positive.

7.6. Glycan isolation (III)

Glycans were detached from cellular glycoproteins by PNGase F digestion (Prozyme, Hayward, CA, USA). First, soluble contaminants were removed by precipitating the proteins with ice-cold 75% ethanol at -20°C. Precipitated proteins were recovered by centrifugation, followed by PNGase F digestion to the protein pellet in 20 mM sodium phosphate buffer pH 7.3 in overnight digestion. The detached glycans then passed in water through Hypersep C_{18} (Thermo Scientific, Waltham, MA, USA) and were absorbed to Hypersep Hypercarb 50mg (Thermo Scientific), both in a 96-well format. The carbon wells were washed with water, and neutral glycans were eluted with 25% acetonitrile in water (v/v); and acidic glycans with 0.05% (v/v) trifluoroacetic acid in 25% acetonitrile in water (v/v). The acidic glycans were further purified by being adsorbed first to MassPREP^{TM} HILIC μElution Plate (Waters, Milford, MA, USA) in 90% acetonitrile, and eluting by 50mM NH_4HCO_3. Both glycan fractions were additionally passed in water through strong cation-exchange resin (Bio-Rad Laboratories, Hercules, CA, USA) and C_{18} silica resin (Millipore, Billerica, MA, USA).

7.7. Mass spectrometry (III)

Mass spectrometry (MS) is based on the separation of ions from a sample, according to their mass-to-charge ratio (m/z), and then recording their relative abundance. First, the compound is transformed into the gas phase by electron ionization. These molecular ions then undergo multiple fragmentations. The ions produced are then separated according to their mass-to-charge ratio and their abundance recorded, producing the mass spectrum of the compound.

7.7.1. MALDI-TOF in this study

Matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry was performed by a Bruker Ultraflex III TOF/TOF instrument (Bruker Daltonics Inc, Bremen, Germany).Acidic N-glycans in negative ion linear mode as $[M - H]^-$ ions and neutral N-glycans were detectable in positive ion reflector mode as $[M + Na]^+$ ions. Relative molar abundances of neutral and acidic glycan components were assigned based on their relative signal intensities in the mass spectra when analyzed separately as the neutral and acidic N-glycan fraction. The mass spectrometric raw data underwent transformation into the present glycan profiles by removal of the effect of isotopic pattern overlapping, multiple alkali-metal adduct signals, products of elimination of water from reducing oligosaccharides, and other interfering mass spectrometric signals not arising from the sample similarly as described (190). Resultant glycan signals in the glycan profiles were normalized to 100% to allow relative quantitative sample comparison. The glycan signals were then assigned to biosynthetic groups based on their proposed monosaccharide composition (120,190), see Supplementary table 1 of Study III.

7.8. Statistical analysis

7.8.1. For mass spectrometry (III)

The mean values of the relative intensities of N-glycan signals of each patient's paired samples served for statistical analyses. Mean relative intensities and the error of means of all N-glycan signals from the whole study group were calculated for neutral and acidic glycans separately. The Mann-Whitney test served to compare differences in glycomic structures between adenomas and carcinomas, and between carcinomas of different stages. When a statistically

significant difference was seen by the Mann-Whitney test, then also the mean of glycan structures' relative amounts, the standard errors of the mean, and the fold change of the means between groups were calculated. Error propagation served to assess standard error for the fold change. For principal component analysis (PCA), the relative intensities of structures were used, whose relative intensities differed significantly, by the Mann-Whitney test, between adenomas and carcinomas. Two components were extracted for both neutral and acidic N-glycans. Bartlett's test showed whether the correlation matrix was identity matrix, and the Kaiser-Meyer-Olking test served to test the adequacy of PCA for the data.

7.8.2. For immunohistochemisty (I-IV)

Immunohistochemical expressions were dichotomized for statistical purposes: PODXL mAb (low vs. high), PODXL pAb (non-membranous vs.membranous), REG4 (negative vs. positive), pauci-mannose (low vs. high), CA19-9 (low vs. high), MUC1 (low vs. high), MUC2 (low vs. high), MUC4 (negative vs. positive), MUC5AC (negative vs. positive), synaptophysin (negative vs. positive), and chromogranin (negative vs. positive).

To study the two PODXL antibodies together required a categorization with three classes: low (mAb=low and pAb=non-membranous), moderate (either mAb=high or pAb=membranous), and high (mAb=high and pAb= membranous). Two similar categorizations were created to analyze REG4 and PODXL together: R+Pm- (REG4=positive and PODXL mAb=low), R-or Pm+ (either REG4=negative or PODXL mAb=high), and R-Pm+ (REG4=negative and PODXL mAb=high). Second category: R+Pp- (REG4=positive and PODXL pAb=non-membranous), R-or Pp+ (either REG4=negative or PODXL

pAb=membranous), and R-Pp+ (REG4=negative and PODXL pAb= membranous).

Evaluation of the association between biomarker expression and clinicopathological parameters was by the exact Pearson chi-square test or the exact linear-by-linear association test for ordered parameters. Disease-specific overall survival was counted from date of surgery to date of death from colorectal cancer, or until end of follow-up. Survival analysis by the Kaplan-Meier method was compared by the log rank test. The Cox regression proportional hazard model served for uni- and multivariable survival analysis, adjusted for sex, age, Dukes classification, and differentiation. Testing of the Cox model assumption of constant hazard ratios over time involved the inclusion of a time-dependent covariate separately for each testable variable. The hazard ratio of differentiation and Dukes D was analyzed in two periods with all biomarkers (0 to 1.25 and 1.25 to 5 years) in order to meet the assumptions of the Cox model, with the time-dependent Cox model. Interaction terms were considered. All tests were two-sided. A p-value of 0.05 was considered significant. All statistical analyses were done with SPSS version 20.0 (IBM SPSS Statistics, version 20.0 for Mac; SPSS, Inc., Chicago, IL, USA)

8. RESULTS

8.1. Neutral N-glycan profiles

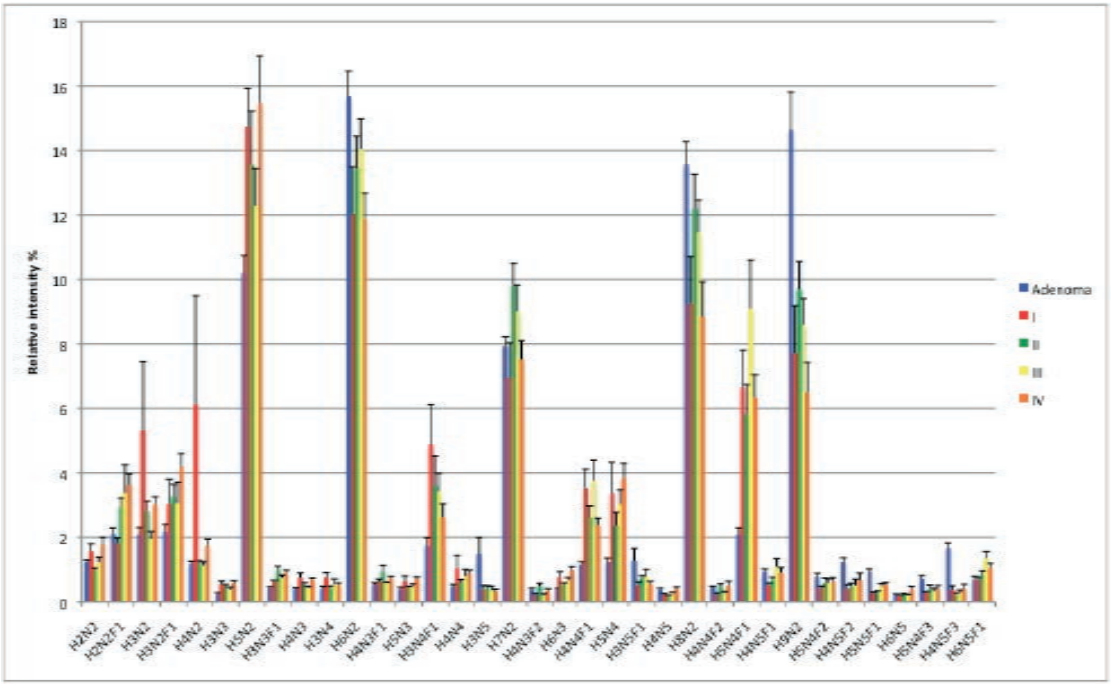
Figure 3A shows an overall comparison of MALDI-TOF mass spectrometric profiles of neutral N-glycans from rectal adenomas and carcinomas. The glycan compositions H5N2, H6N2, H7N2, H8N2 and H9N2, identified as high-mannose type-N glycans, were the most abundant glycan signals of the neutral N-glycan fractions of both adenomas and carcinomas. However, in comparison to adenomas, the relative amount of the smallest high-mannose type glycan H5N2 was higher in all carcinoma stages compared to the other high-mannose glycans. Neutral complex-type N-glycans, most notably H3N4F1, H4N4F1, H5N4, and H5N4F1, were more abundant in carcinomas than in adenomas. The third notable group of neutral glycan signals in the present study was of pauci-mannose type N-glycans, which were more abundant in carcinomas than in adenomas, glycans such as H2N2F1, H3N2, H3N2F1, and H4N2.

8.2. Acidic N-glycan profiles

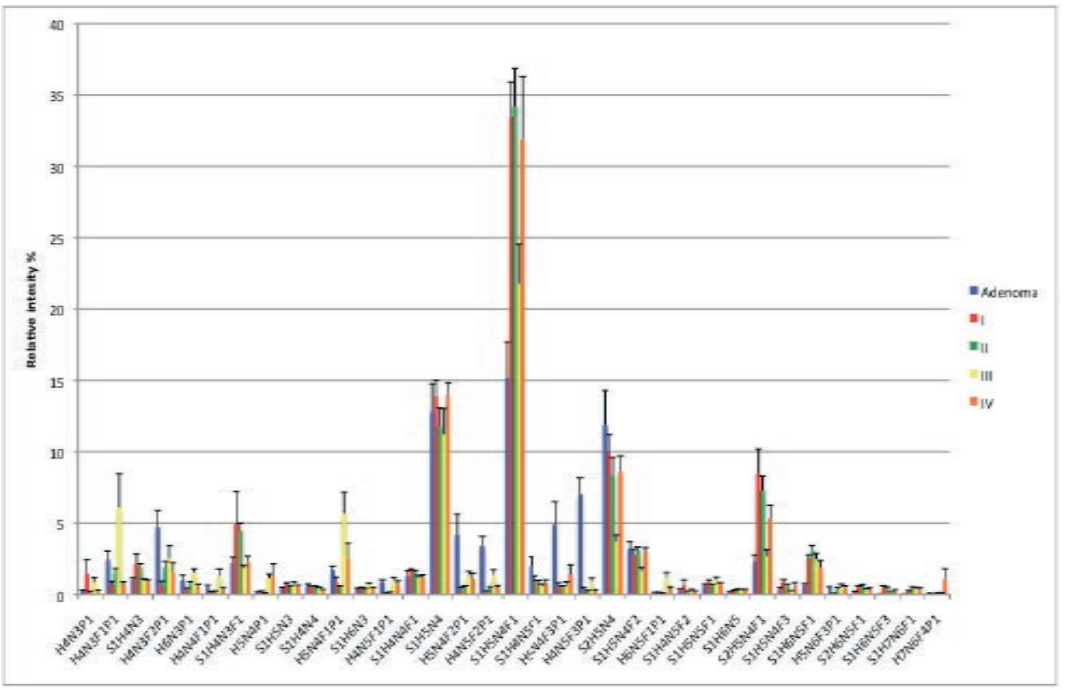
Figure 3B shows the overall acidic N-glycan profiles of the sample group. In the acidic N-glycan profiles, the acid ester-containing structures were major glycans in adenomas but not in carcinomas, ones such as H5N4F2P1, H4N5F2P1, H5N4F3P1, and H4N5F3P1. Sialylated structures dominated the carcinoma acidic N-glycan profiles, for example the sialylated N-glycans S1H5N4F1 and S2H5N4F1. Larger sialylated N-glycans like S1H6N5F1 were more common in carcinomas than in adenomas.

Figure 3. Neutral (A) and acidic (B) N-glycan profile of rectal adenomas and carcinomas of different stages

A



B



The relative intensities of the 35 most abundant glycan signals of rectal adenomas and carcinomas of different stages (I-IV). Error bars represent error of means. All glycan signals have been assigned to proposed monosaccharide compositions (see Abbreviations). Neutral glycan signals were analyzed as sodium adduct ions, [M+Na]+ and acidic glycan signals as deprotonated ions, [M-H]-. Major N-glycans are described with symbol methodology based on previous structural analyses. Brackets indicate that position of the acid ester (SP) in the structure is not specified. Blue square= N-acetylhexosamine, green circle=, hexose, red triangle = deoxyhexose/fucose, yellow circle=mannose, purple diamond=N-acetylneuraminic acid . Adapted from Study III, permission by CC BY.

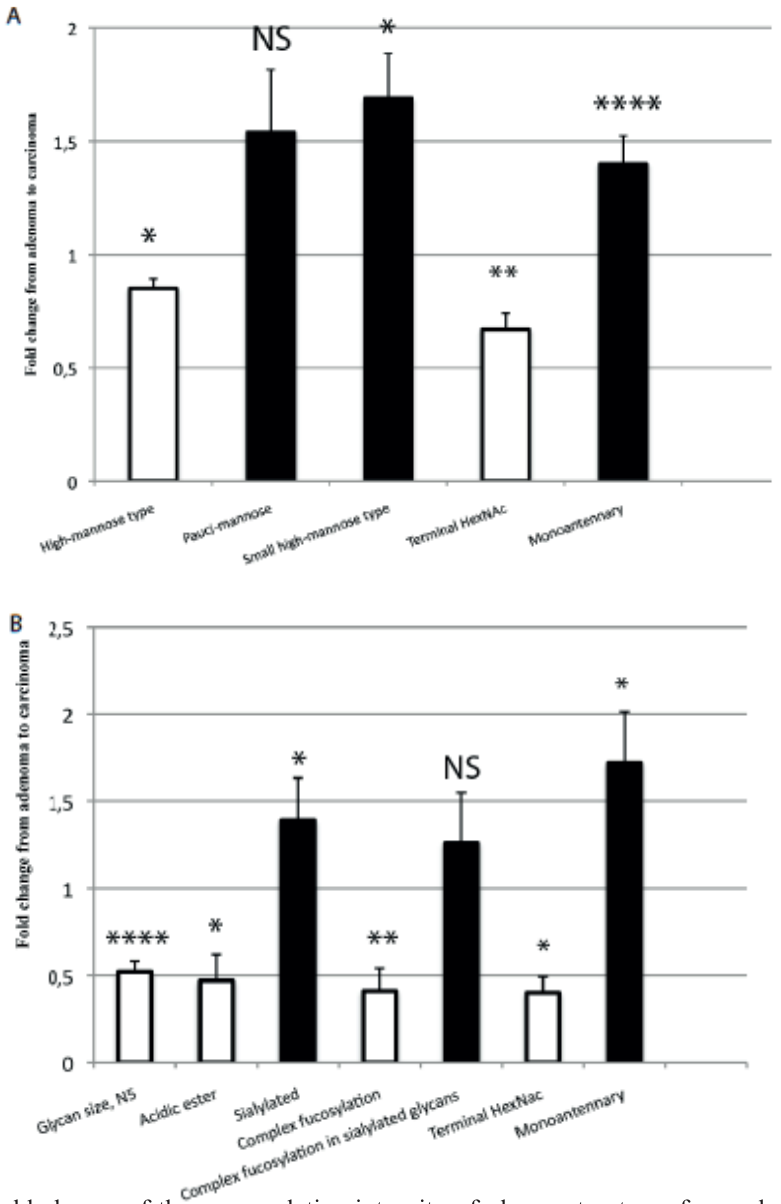
8.3. Glycosylation differences between adenomas and carcinomas

Statistically significant changes in N-glycan structures were evident between adenomas and carcinomas (Figure 4A+B). A significant increase occurred in monoantennary-size structures for both acidic and neutral N-glycans in carcinomas compared to levels in adenomas.

Sialylated structures were more common in carcinomas, whereas acid ester-modified N-glycans were especially abundant in adenomas. Neutral complex- and hybrid-type N-glycans were also more common in carcinomas as evidenced in their significantly increased relative presence in the neutral N-glycan fraction. In carcinomas, we saw an increase, although statistically non-significant, of pauci-mannose structures among neutral N-glycans. Glycan signals that could potentially contain sialylated Lewis-type structures were also higher in carcinomas than in adenomas, although this difference was statistically non-significant. All p-values and ratios of means are in Supplementary Table 2A and B of Study III.

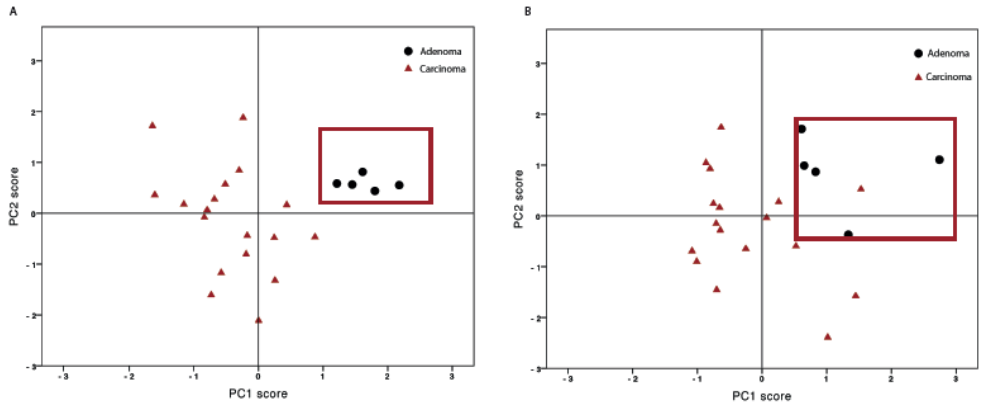
Principal component analysis, when the structures with statistically significant differences between adenomas and carcinomas (5 structure types in neutral N-glycans and 6 in acidic N-glycan structures) were included, demonstrated that neutral N-glycans were homogenous in adenomas, but not in carcinomas. The profiles of carcinomas differed from those of adenomas, but they also differed among themselves (Fig 5A). A similar phenomenon was apparent for acidic N-glycan profiles (Fig 5B).

Figure 4. Differences in neutral (A) and acidic (B) N-glycosylation between adenomas and carcinomas



Fold change of the mean relative intensity of glycan structures from adenomas to carcinomas. Statistical analysis by the Mann-Whitney-test: *=($p<0.05$), **=($p<0.01$), ***=($p<0.001$), ****=($p<0.0001$), NS=Non-significant. Adapted from publication III, permission by CC BY.

Figure 5. Principal component analysis separates rectal adenomas and carcinomas based on neutral (A) and acidic (B) N-glycosylation



For neutral glycans (Bartlett's test: p=0.005, Kaiser-Meyer-Olking test: 0.588, PC1 (42.5%) vs PC2 (25.6%)). For acidic glycans (Bartlett's test: p<0.00001, Kaiser-Meyer-Olking test: 0.741, PC1 (83.3%) vs PC2 (8.1%). Adapted from publication III, permission by CC BY.

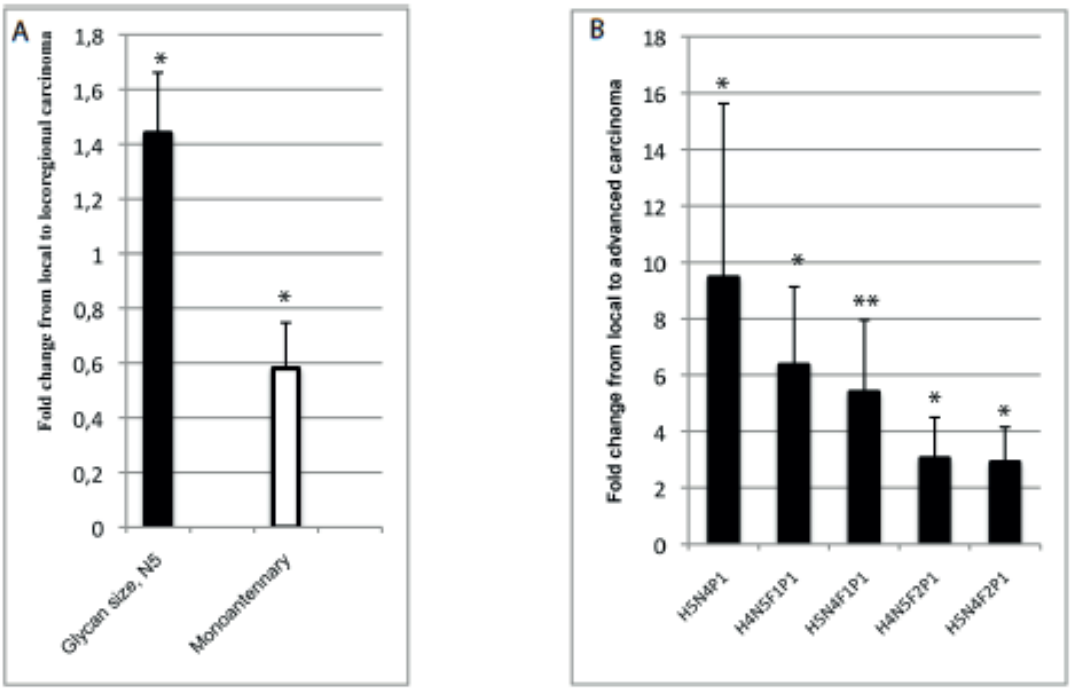
8.4. Glycosylation differences between local disease and locoregional disease

Acidic N-glycan signals with five N-acetylhexosamine residues were significantly more abundant in stage III carcinoma than in stage I-II carcinomas, whereas monoantennary-size N-glycans were more abundant in local disease samples than in those locoregional (Fig 6A). P-values and ratios of means are in Supplementary Table 2C of Study III.

8.5. Glycosylation differences between local disease and advanced disease

Acid ester-modified N-glycans, namely H5N4P1, H5N4F1P1, H5N4F2P1, H4N5F1P1, and H4N5F2P1, were increased in stage III-IV carcinomas compared to stage I-II carcinomas (Fig. 6B). P-values and ratios of means are in Supplementary Table 2D of Study III.

Figure 6. Differences in acidic N-glycosylation between (A) local and locoregional rectal carcinoma, (B) local and advanced rectal carcinoma



Fold change of the mean relative intensity of glycan structures from (A) local (stage I-II) to locoregional carcinomas (stage III), and local to advanced carcinoma (stage III-IV). Statistical analysis by the Mann-Whitney-test: *=($p<0.05$), **=($p<0.01$), ***=($p<0.001$), ****=($p<0.0001$), NS=Non-significant. Adapted from publication III, permission by CC BY.

8.6. Immunohistochemistry (I-IV)

With the PODXL mAb , PODXL stained evenly throughout the cytoplasm in a granular manner in the vast majority of tumours, and staining was visible in all tumour cells. Neither nuclear nor cell membranous immunopositivity occurred. PODXL expression by the pAb was cytoplasmic in tumour cells, but in some cases a distinct membranous expression was visible, which did not always correlate with intensity of cytoplasmic expression.

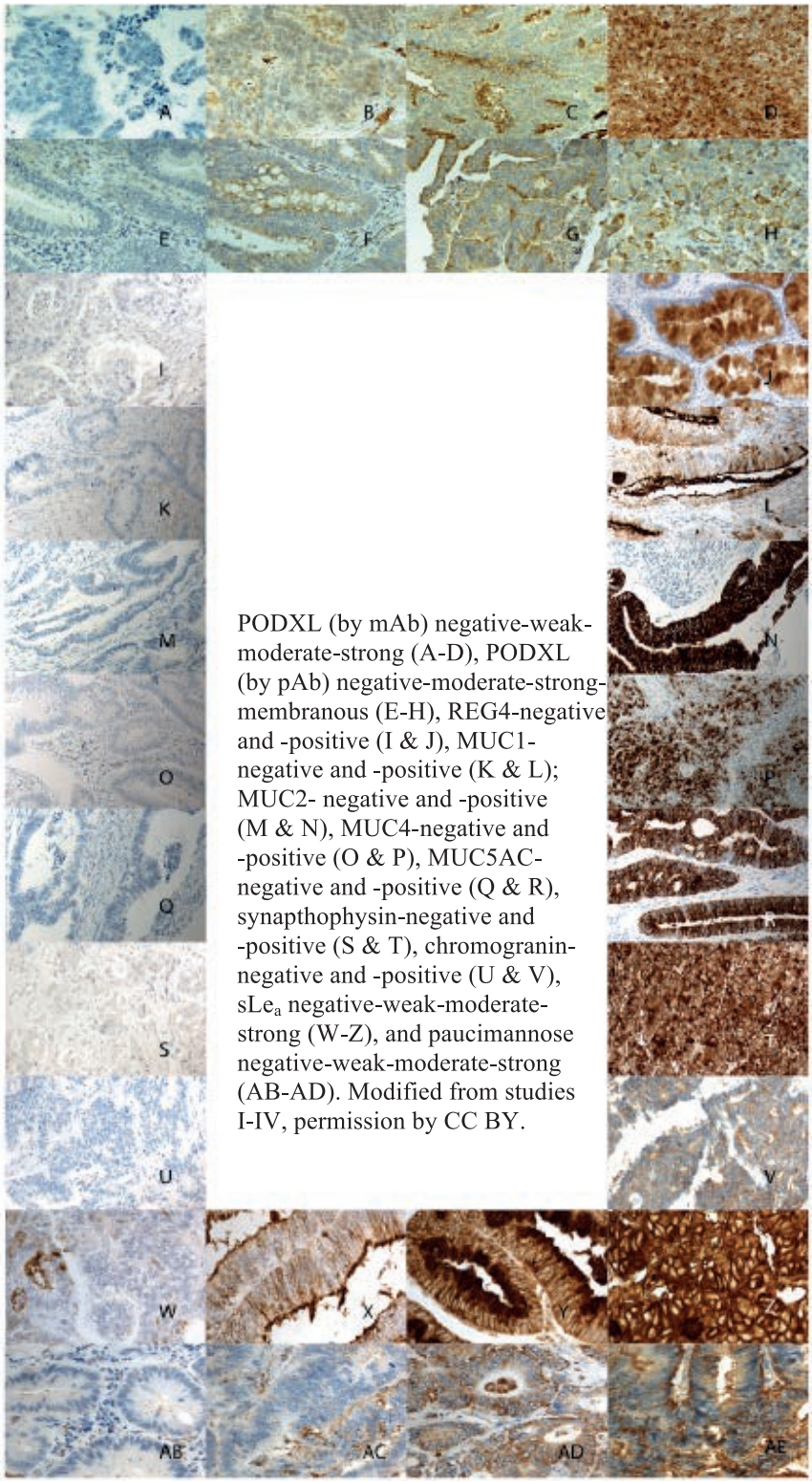
REG4 expression in tumour cells was cytoplasmic and slightly granular. When present, expression was evident in the vast majority of tumour cells, but with no nuclear expression. In whole-tissue sections, no clear distinction in expression appeared between the invasive front and the rest of the tumour. Moreover, in whole sections, REG4 expression appeared in some cases in normal epithelium, but was down-regulated in tumour cells. Expression of mucins, synapthopysin, and chromogranin was cytoplasmic, with no nuclear expression.

sLe_a expression was membranous with apical membrane predilection and partially cytoplasmic, but with no visible nuclear staining. Pauci-mannose expression was mostly cytoplasmic and often granular, with only minor membranous accumulation and no visible nuclear staining. Expression of each is in Table 6 and representative images are in Figure 8.

Table 6. Immunohistochemical expression of biomarkers in colorectal cancer

Marker	**Patients**	**N(%)**			
PODXL mAb		**Negative**	**Weak**	**Moderate**	**Strong**
	767	41 (5.3)	430 (56.1)	252 (32.9)	44 (5.7)
PODXL pAb		**Negative**	**Moderate**	**Strong**	**Membranous**
	780	46 (5.9%)	322 (41.2%)	349 (44.7%)	63 (8.1)
REG4		**Negative**			**Positive**
	793	580 (73.1)			213 (28.9)
MUC1		**Negative**	**Weak**	**Moderate**	**Strong**
	206	80 (38.8)	90 (43.7)	27 (13.1)	9 (4.4)
MUC2		**Negative**	**Weak**	**Moderate**	**Strong**
	210	143 (68.1)	31 (14.8)	19 (9.0)	17 (8.1)
MUC4		**Negative**	**Weak**	**Moderate**	**Strong**
	208	106 (51.0)	77 (37.0)	17 (8.2)	8 (3.8)
MUC5AC		**Negative**	**Weak**	**Moderate**	**Strong**
	205	191 (93.2)	9 (4.4)	1 (0.4)	4 (2.0)
Synaptophysin		**Negative**			**Positive**
	215	209 (97.2)			6 (2.8)
Chromogranin		**Negative**			**Positive**
	217	212 (97.7)			9 (2.3)
Paucimannose		**Negative**	**Weak**	**Moderate**	**Strong**
	208	8 (3.8)	107 (51.4)	76 (36.5)	17 (8.2)
sLe_a		**Negative**	**Weak**	**Moderate**	**Strong**
	215	20 (9.3)	47 (21.9)	76 (35.3)	72 (33.5)

Figure 7. Representative images of antibody stainings in colorectal cancer



PODXL (by mAb) negative-weak-moderate-strong (A-D), PODXL (by pAb) negative-moderate-strong-membranous (E-H), REG4-negative and -positive (I & J), MUC1-negative and -positive (K & L); MUC2- negative and -positive (M & N), MUC4-negative and -positive (O & P), MUC5AC-negative and -positive (Q & R), synapthophysin-negative and -positive (S & T), chromogranin-negative and -positive (U & V), sLe_a negative-weak-moderate-strong (W-Z), and paucimannose negative-weak-moderate-strong (AB-AD). Modified from studies I-IV, permission by CC BY.

Table 7. Association of PODXL with clinicopathological variables by mAb HES9, pAb HPA002110, and their combination in CRC

	PODXL expression by mAb			PODXL expression by pAb			Combined			
n (%)	Low 723 (94.3)	High 44 (5.7)	p-value*	Non-membranous 717 (91.9)	Membranous 63 (8.1)	p-value*	Low 714 (88.4)	Moderate 79 (9.8)	High 14 (1.7)	p-value*
Age, years										
<65	309 (42.7)	16 (36.4)	0.436	299 (41.7)	32 (50.8)	0.184	300 (42.0)	38 (48.1)	5 (35.7)	0.643
≥65	414 (57.3)	28 (63.6)		418 (48.3)	31 (49.2)		414 (58.0)	41 (51.9)	9 (64.3)	
Gender										
Male	402 (44.4)	24 (54.5)	1.000	398 (55.5)	31 (49.2)	0.357	398 (55.7)	41 (51.9)	7 (50.0)	0.450
Female	321 (55.6)	20 (45.5)		319 (44.5)	32 (50.8)		316 (44.3)	38 (48.1)	7 (50.0)	
Dukes										
A	109 (15.1)	2 (4.5)	**0.012**	111 (15.5)	2 (3.2)	**<0.001**	113 (15.8)	4 (5.1)	0 (0.0)	<0.0001
B	259 (35.8)	13 (29.5)		258 (36.0)	17 (27.0)		257 (36.0)	26 (32.9)	2 (14.3)	
C	196 (27.1)	14 (31.8)		191 (26.6)	22 (34.9)		187 (26.2)	32 (40.5)	2 (14.3)	
D	159 (22.0)	15 (34.1)		157 (21.9)	22 (34.9)		157 (22.0)	17 (21.5)	10 (71.4)	
Grade (WHO)										
1	26 (3.6)	0 (0)	**<0.0001**	27 (3.8)	0 (0)	**<0.0001**	28 (3.9)	0 (0.0)	0	**<0.0001**
2	514 (71.5)	11 (25.0)		511 (71.8)	27 (42.9)		513 (72.4)	32 (40.5)	3 (21.4)	
3	161 (22.4)	25 (56.8)		155 (21.8)	30 (47.6)		149 (21.0)	37 (46.8)	9 (64.3)	
4	18 (2.5)	8 (18.2)		19 (2.7)	6 (9.5)		19 (2.7)	10 (12.7)	2 (14.3)	
Missing	4			5			5			
Location										
Colon	372 (51.5)	28 (63.6)	0.123	370 (51.6)	35 (55.6)	0.548	362 (50.7)	49 (62.0)	7 (50.0)	0.168
Rectum	351 (48.5)	16(36.3)		347 (48.4)	28 (44.4)		352 (49.3)	30 (38.0)	7 (50.0)	
Side										
Right	189 (26.1)	23 (52.3)	**<0.001**	193 (26.9)	21 (33.3)	0.274	185 (25.9)	32 (40.5)	6 (42.9)	**0.003**
Left	534 (73.9)	21 (47.7)		542 (73.1)	42 (66.7)		529 (74.1)	47 (59.5)	8 (57.1)	
Histology										
Adenomatous	648 (89.8)	39 (88.6)	0.798	644 (89.9)	57 (90.5)	1.000	639 (89.6)	70 (88.6)	13 (92.9)	0.879
Mucinous	74 (10.2)	5 (11.4)		72 (10.1)	6 (9.5)		74 (10.4)	9 (11.4)	1 (7.1)	
Missing	1			1			1			

*By Pearson chi-square exact-test or linear-by-linear test for ordered parameters. Missing data not included in the analyses. Modified from publications I-II, permission by CC BY.

Table 8. Association of REG4, Pauci mannose, and sLe_a with clinicopathological variables in CRC

	REG4			Pauci mannose			sLe_a		
n (%)	negative 580 (73.1)	positive 213 (28.9)	p-value*	Low 115(55.3)	High 93(44.7)	p-value*	Low 143(66.5)	High 72(33.5)	p-value*
Age, years									
<65	245 (42.2)	94 (44.1)	0.686	43(46.2)	42(45.2)	0.894	63(44.1)	36(50.0)	0.409
≥65	335 (57.8)	119 (55.9)		62(53.8)	51(54.8)		80(55.9)	36(50.0)	
Gender									
Male	313 (54.0)	128 (60.1)	0.124	72(62.6)	55(59.1)	0.610	86(60.1)	45(62.5)	0.738
Female	267 (46.0)	85 (39.9)		43(37.4)	38(40.9)		57(39.9)	27(37.5)	
Dukes									
A	75 (12.9)	44 (20.7)	**0.014**	19 (16.5)	12 (12.9)	0.182	25 (17.5)	9 (12.5)	0.104
B	202 (34.8)	73 (34.3)		34 (29.6)	37 (39.8)		51 (35.7)	20 (25.8)	
C	164 (28.3)	55 (25.8)		43 (37.4)	24 (25.8)		42 (29.4)	26 (36.1)	
D	139 (24.0)	41 (19.2)		19 (16.5)	20 (21.8)		25 (17.5)	17 (23.6)	
Grade (WHO)									
1	14 (2.4)	13 (6.2)	0.064	4 (3.5)	3 (3.3)	0.891	6 (4.3)	2 (2.9)	**0.017**
2	394 (68.3)	150 (71.1)		87 (76.3)	69 /76.7)		113 (80.7)	49 (70.0)	
3	150 (26.0)	38 (18.0)		20 (17.5)	17 (18.9)		21(15.0)	16 822.9)	
4	19 (3.3)	10 (4.7)		3 (2.6)	1 (1.1)		0 (0)	3 (4.3)	
Missing	3	2		1	3		3	2	
Location									
Colon	294 (50.7)	116 (54.5)	0.346	45(39.1)	40(43.0)	0.571	59(41.3)	25(34.7)	0.354
Rectum	286 (49.3)	97 (45.5)		70(60.9)	53(57.0)		84(58.7)	47(65.3)	
Side									
Right	147 (25.3)	70 (32.9)	**0.035**	30(26.1)	20(21.5)	0.442	35(24.5)	14(19.4)	0.407
Left	433 (74.7)	143 (67.1)		85(73.9)	73(78.5)		108(75.5)	58(80.6)	
Histology									
Adenomatous	539 (92.9)	173 (81.6)	**<0.0001**	104(91.2)	92(98.9)	**0.014**	137(95.8)	66(93.0)	0.375
Mucinous	41 (7.1)	39 (18.4)		10(8.8)	1(1.1)		6(4.2)	5(7.0)	
Missing				1				1	

*By Pearson chi-square exact-test or linear-by-linear test for ordered parameters. Missing data not included in the analyses. Modified from publications III-IV, permission by CC BY.

8.7. Expression patterns of the two PODXL antibodies

The agreement of expression of the two antibodies across cases was low (kappa-value=0.219, standard error 0.060, $p<0.0001$) using dichotomous values for both antibodies. The distinctive strong staining by mAb (n=44) and membranous staining by pAb (n=63) was shared by only 14 tumours.

8.8. Association with clinicopathological variables (I-IV)

The associations of PODXL, REG4, Pauci-mannose, and sialyl Lewis with clinicopathological paramateters were done by the exact Pearson test of linear-by-linear test for ordered parameters (Tables 7 and 8). High cytoplasmic expression of PODXL by the mAb associated with low differentiation, advanced disease, and location in the right hemicolon. Results were similar for the membranous expression of PODXL by the pAb, but no association with right hemicolon was apparent. The combined expression of both PODXL antibodies yielded results similar to those of the mAb.

Positive cytoplasmic REG4 associated with local disease, mucinous histology, and location of the tumour in the right hemicolon. Cytoplasmic pauci-mannose expression associated with non-mucinous histology. sLe_a associated with poor differentiation.

8.9. Association of REG4 with other biomarkers (IV)

In the subgroup of 220 tumours, we found that REG4 expression significantly associated with higher expression of MUC2, MUC4, and MUC5AC, but not

with MUC1 expression. Nor did REG4 expression associate with markers of neuroendocrine differentiation (Table 9).

Table 9. REG4 associates with MUC1, MUC2, and MUC4 expression in CRC

	REG4		
	negative	**positive**	
n (%)	**162 (76.4)**	**50 (23.6)**	**p-value***
MUC1 expression			
low	131 (84.0)	36 (78.3)	0.368
high	25 (16.0)	10 (21.7)	
MUC2 expression			
low	147 (93.6)	24 (48.0)	**<0.0001**
high	10 (6.4)	26 (52.0)	
MUC4 expression			
negative	90 (57.0)	14 (29.2)	**0.001**
positive	68 (43.0)	34 (70.8)	
MUC5AC expression			
negative	150 (96.2)	38 (82.6)	**0.004**
positive	6 (3.8)	8 (17.4)	
Neuroendocrine differentiation			
negative	152 (93.8)	45 (90.0)	0.354
positive	10 (6.2)	5 (10.0)	

*By Pearson chi-square exact-test or linear-by-linear test for ordered parameters. Missing data is not included in the analyses. Modified from publication IV, permission by CC BY.

8.10. Association of PODXL with REG4 in CRC (unpublished data)

Positive REG4 expression was associated with non-membranous PODXL expression by the pAb, but no association was apparent by PODXL expression by the mAb. With the combined PODXL expression, low expression associated with REG4 positivity. (Table 10)

Table 10. Association of REG4 with PODXL in CRC

	REG4 Expression		
	negative	**positive**	
n (%)	**580 (73.1)**	**213 (28.9)**	**p-value***
PODXL expression			
Low	506 (93.5)	188 (95.9)	0.22
High	35 (6.5)	8 (6.1)	
missing	39	17	
PODXL expression			
Non-membranous	502 (90.3)	197 (96.1)	**0.010**
Membranous	54 (9.7)	8 (3.9)	
Missing	24	8	
Combined PODXL			
Low	489 (86.4)	195 (92.9)	**0.012**
Moderate	65 (11.5)	14 (6.7)	
High	12 (2.1)	1 (0.5)	
Missing	14	3	

*By Pearson chi-square exact-test or linear-by-linear test for ordered parameters. Missing data is not included in the analyses

8.11. Univariable survival analysis

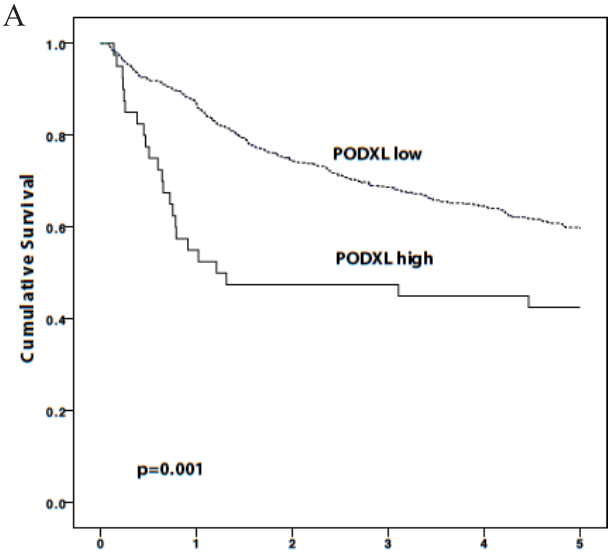
8.11.1. Podocalyxin

In CRC, Kaplan-Meier analysis showed significantly poorer disease-specific survival (DSS) for patients with high PODXL (by the mAb) expression (p=0.001) (Figure 8A). Five-year DSS was 42.5% for high PODXL expression (95% CI 27.2-57.8%) and 59.8% (95% CI 56.1-63.5%) for low expression. Because of the difference in the biological and anatomical background of the right and left colorectum, and also in expression pattern of PODXL (by mAb) results were also stratified for RHC vs. LHC. For RHC cancer patients, no evidence of any difference in survival emerged between patients with high

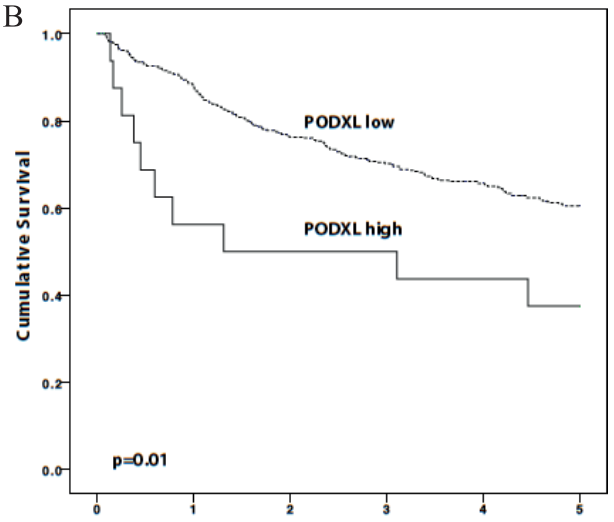
compared to low PODXL expression (p=0.148) LHC cancer patients with high PODXL expression had significantly poorer DSS than did those with low expression. (p=0.01) (Figure 8B). Five-year DSS for LHCC patients was 33.3% (95% CI 11.5-55.1%) for high vs. 60.2% (95% CI 55.9-64.5%) for low PODXL tumour expression.

Figure 8. Five-year DSS according to PODXL expression by mAb HES9 in colorectal cancer (A) and cancer of the left hemicolon (B). Log-rank test used here.

A

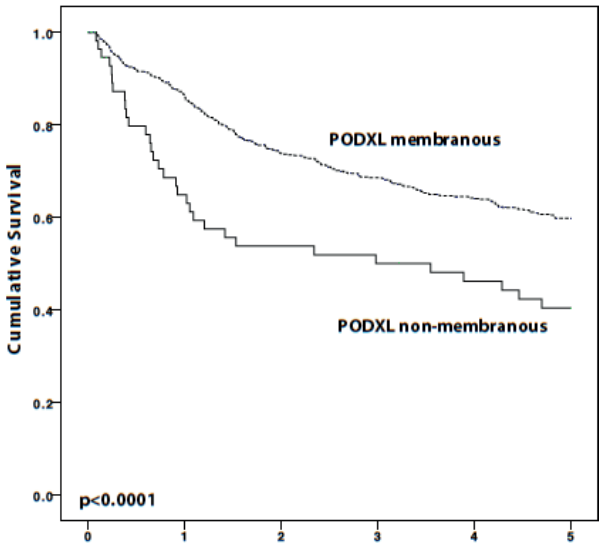


B



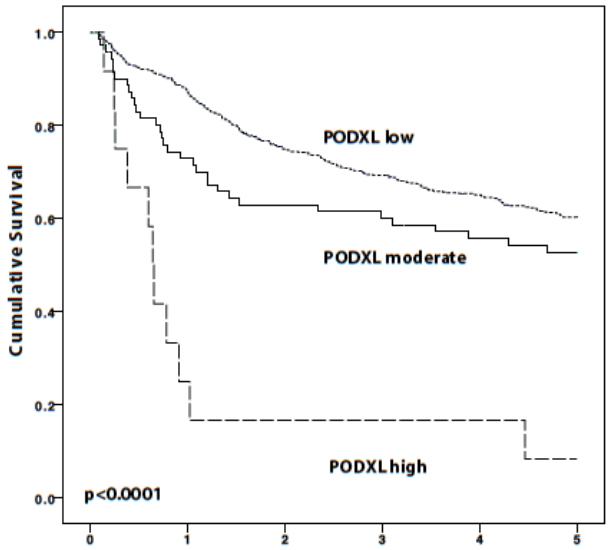
PODXL expression by the pAb showed that colorectal cancer patients with membranous PODXL expression disease-specific survival (DSS) was significantly poorer (p=0.0001) (Figure 9). Five-year DSS was 40.5% (95% CI 27.4-53.6%) for patients with membranous PODXL expression compared to 60.0% (95% CI 56.3-63.7%) for non-membranous expression.

Figure 9. Five-year DSS according to PODXL expression by pAb HPA002110 in colorectal cancer, by log-rank test.



Because the two PODXL antibodies recognised two different groups of patients with a poor prognosis, a combination of their expression required investigation. Combination (low-moderate-high) mAb and pAb showed a significantly poorer DSS for colorectal cancer patients with high expression than with low ($p<0.0001$) or moderate expression ($p<0.0001$). No statistically significant difference in DSS was evident between those with low and moderate expression (p=0.083). Five-year DSS for CRC patients with low expression was 60.3% (95% CI 56.6-64.0%), for those with moderate expression 52.8% (95% CI 41.0-64.6%), and for those with high expression 8.3% (95% CI -7.4-24.0%).

Figure 10. Five-year DSS according to combined PODXL expression by the mAb HES9 and pAb HPA002110 in colorectal cancer by the global log-rank test.

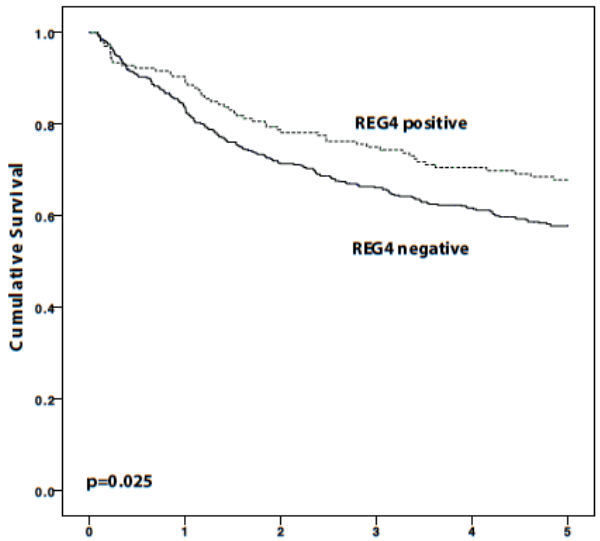


8.11.2. REG4

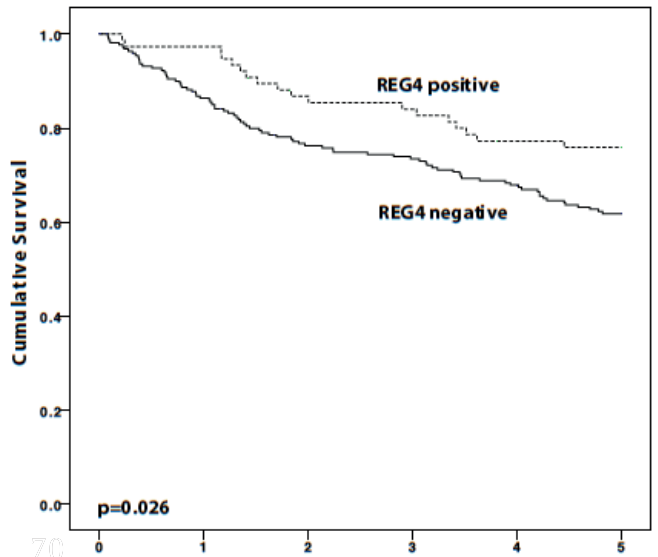
Since REG4 expression differed between non-mucinous and mucinous CRC, a analysis was done for them separately. In non-mucinous CRC REG4 positivity was a sign of favourable prognosis (p=0.025); 5-year DSS for patients with positive cytoplasmic REG4 tumour expression was 67.9% (95% CI 60.5-75.3) compared to 57.8% (95%CI 53.5-62.1) for those with no cytoplasmic expression (Fig 11A). In mucinous CRC, no difference appeared (data not shown). Because an interaction occurred between REG4 expression and age, a further stratification was done for non-mucinous CRC for patients under or over 65. REG4 expression was a sign of favourable prognosis in patients under 65 (p=0.026) (Fig 11B), with no difference apparent for those older (p=0.342).

Figure 11. Five-year DSS according to REG4 expression in non-mucinous CRC (A) and in non-mucinous CRC for patients under 65 (B), by log-rank test.

A



B



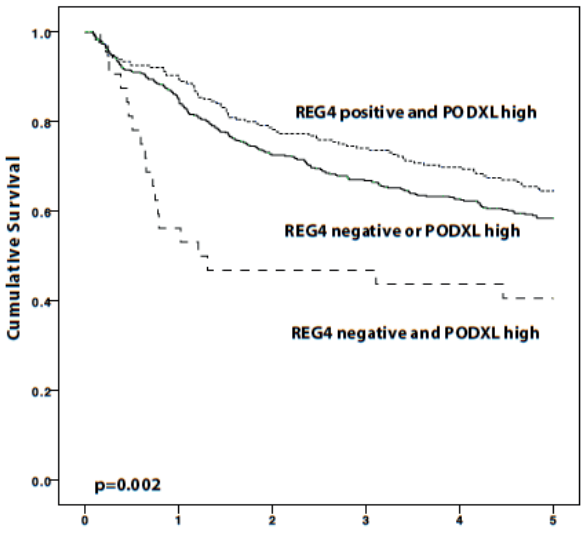
8.11.3. PODXL and REG4 combined

CRC patients with with concomitant negative REG4 expression and high PODXL expression (by mAb) had a poorer 5-year DSS than did the (R+Pm-) group ($p<0.0001$) and (R-or Pm-) group ($p=0.005$). No difference appeared between the latter two groups ($p=0.092$). The 5-year DSS was: (R-Pm+) group 40.6% (95%CI 23.6-57.7), (R-or Pm-) group 58.5% (95%CI 54.2-62.8), and (R+Pm-) group 64.4% (95%CI 58.1-70.7).

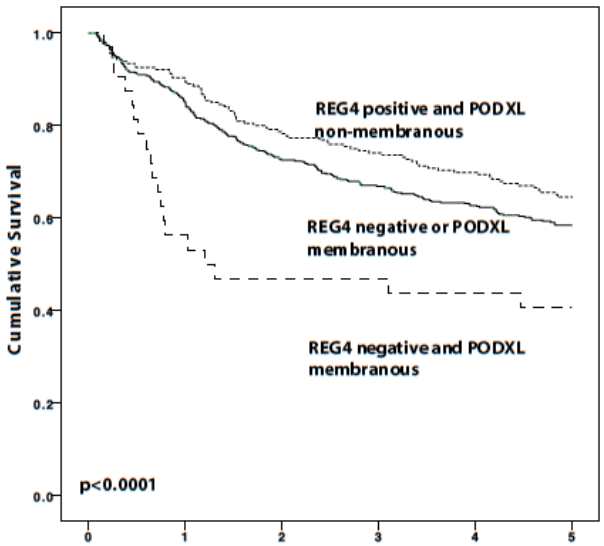
A similar effect was seen with concomitant negative REG4 expression and membranous PODXL expression (by pAb) compared to the (R+Pp-) group ($p<0.0.0001$) and (R-or Pp+) group ($p=0.001$). No difference appeared between the latter two groups ($p=0.24$). The 5-year DSS were: (R-Pp+)-group 37.3% (95%CI 23.0-51.6), (R-or Pp-) group 59.3% (95%CI 55.0-63.6), and (R+Pp-) group 63.7% (95%CI 57.2-70.2).

Figure 12. Five-year DSS according to concomitant expression (A) by REG4 and PODXL (mAb) and REG4 and PODXL (pAb), by global log-rank test.

A



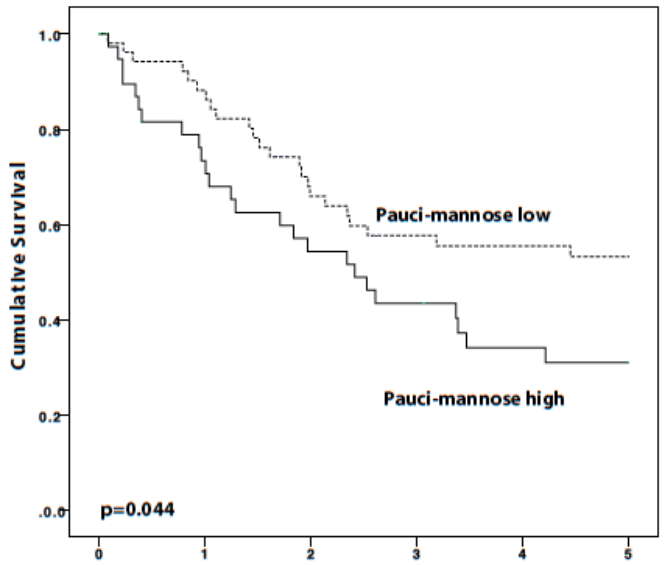
B



8.11.4. Pauci mannose

Five-year DSS was significantly poorer for patients with advanced colorectal cancer with high pauci-mannose expression (p=0.044), being 31.1% (95% CI 15.8-46.4%), than for those with low expression, 55.7% (95% CI 42.4-69.0%). Fig 13. When the all stages were included, no difference emerged in survival (data not shown).

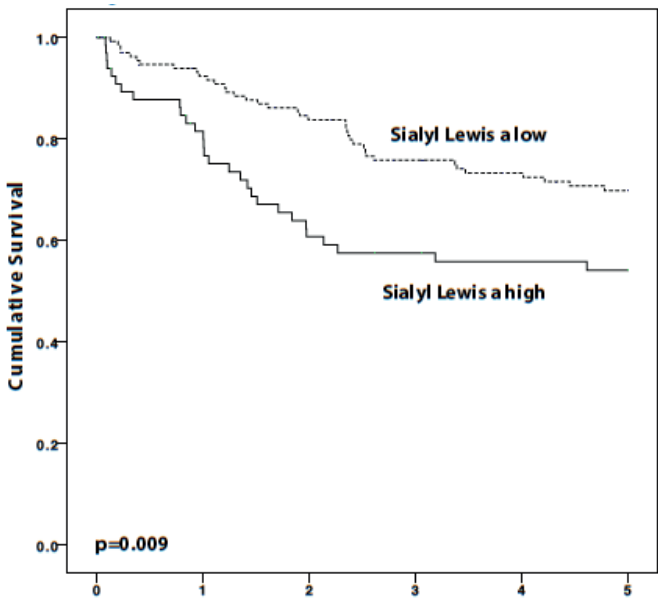
Figure 13. Five-year DSS according to pauci-mannose expression in colorectal cancer. Log-rank test was used here.



8.11.5. Sialyl Lewis a

Five-year DSS of CRC patients with high sLe_a expression was poorer than for patients with low sLe_a expression ($p<0.009$), being 42.5% (95% CI 27.2-57.8%) for high expression and 59.8% (95% CI 56.1-63.5%) for low expression. Fig.14

Figure 14. Five-year DSS according to sLe_a expression in colorectal cancer. Log-rank test was used here.



8.12. Multivariable survival analysis

A Cox proportional hazard model adjusted for sex, age, Dukes classification, and differentiation served for multivariable survival analysis. In the multivariable models, higher stage, poorer differentiation, and age over 65 were independent markers of poor prognosis.

High PODXL by the mAB (Table 11A) and membranous PODXL expression by the pAb (Table 11B) were independent markers of poor prognosis in CRC (respectively ,HR 1.82, 95% CI 1.15-2.86, p=0.01; and HR 1.64, 95% CI 1.11-2.43, p=0.012). Their combination was also an independent marker of poor prognosis (moderate vs low HR 1.63, 95% 1.11-2.39, p=0.012; and high vs low HR 2.14, 95% CI 1.12-4.07, p=0.021) (Table 11C).

For non-mucinous-CRC patients under age 65, REG4 was an independent factor of favourable prognosis (HR 0.55, 95% CI 0.33-0.92, p=0.022) (Table 11D).

High CA19-9 expression was an independent marker of poor prognosis in CRC (HR 1.80, 95% CI 1.09-2.98, p=0.023) (Table 11E).

Table 11.A Cox multivariable analysis of relative risk of death from non-mucinous colorectal cancer within 5 years by REG4 expression for patients under 65

	HR (95% CI)	p-value
REG4 expression		
Negative	1.00	
Positive	0.55 (0.33-0.92)	**0.022**
Gender		
Female	1.00	
Male	0.97 (0.65-1.44)	0.865
Stage		
Dukes A	1.00	
Dukes B	2.38 (0.68-8.37)	0.175
Dukes C	6.16 (1.88-20.19	**0.003**
Dukes D	30.65 (9.52-98.69)	**<0.0001**
Differentiation		
Grades 1-2	1.00	
Grades 3-4 (0-1.25y)	2.45 (1.32-4.55)	**0.005**
Grades 3-4 (1.25-5.0y)	0.31 (0.13-0.76)	**0.010**

Abbreviations CI=Confidence interval, HR=Hazard ratio

Table 11.B Cox multivariable analysis of relative risk of death from colorectal cancer within 5 years by PODXL expression by mAb HES9

	HR (95% CI)	p-value
PODXL expression		
Low	1.00	
High	1.82 (1.15-2.86)	**0.01**
Age		
≤65	1.00	
>65	1.75 (1.38-2.23)	**<0.0001**
Gender		
Female	1.00	
Male	1.04 (0.82-1.31)	0.759
Stage		
Dukes A	1.00	
Dukes B	2.51 (1.24-5.10)	**0.011**
Dukes C	6.75 (3.38-13.46)	**<0.0001**
Dukes D (0-1.25y)	44.47 (21.22-93.18)	**<0.0001**
Dukes D (1.25-1.5y)	25.91 (12.54-53.54)	**<0.0001**
Differentiation		
Grades 1-2	1.00	
Grades 3-4 (0-1.25y)	1.92 (1.36-2.70)	**<0.0001**
Grades 3-4 (1.25-5.0y)	0.801 (0.53-1.20)	0.285

Abbreviations CI=Confidence interval, HR=Hazard ratio

Table 11.C Cox multivariable analysis of relative risk of death from colorectal cancer within 5 years by PODXL expression by pAb HPA002110

	HR (95% CI)	p-value
PODXL expression		
Non-membranous	1.00	
Membranous	1.64 (1.11-2.43)	**0.012**
Age		
≤65	1.00	
>65	1.98 (1.56-2.52)	**<0.0001**
Gender		
Female	1.00	
Male	1.01 (0.80-1.27)	0.925
Stage		
Dukes A	1.00	
Dukes B	2.86 (1.36-6.01)	**0.006**
Dukes C	7.84 (3.79-16.22)	**<0.0001**
Dukes D (0-1.25y)	51.173 (23.61-110.91)	**<0.0001**
Dukes D (1.25-1.5y)	31.398 (14.65-67.28)	**<0.0001**
Differentiation		
Grades 1-2	1.00	
Grades 3-4 (0-1.25y)	2.13 (1.52-2.98)	**<0.0001**
Grades 3-4(1.25-5.0y)	0.92 (0.62-1.37)	0.684

Abbreviations CI=Confidence interval, HR=Hazard ratio

Table 11.D Cox multivariable analysis of relative risk of death from colorectal cancer within 5 years by combined PODXL expression by mAb HES9 and pAb HPA002110

	HR (95% CI)	p-value
PODXL expression		
Low	1.00	
Moderate	1.63 (1.11-2.39)	**0.012**
High	2.14 (1.12-4.07)	**0.021**
Age		
≤65	1.00	
>65	1.89 (1.50-2.40)	**<0.001**
Gender		
Female	1.00	
Male	1.06 (0.85-1.33)	0.601
Stage		
Dukes A	1.00	
Dukes B	2.60 (1.29-5.27)	**0.008**
Dukes C	7.01 (3.56-14.14)	**<0.0001**
Dukes D (0-1.25y)	46.46 (22.21-97.16)	**<0.0001**
Dukes D (1.25-1.5y)	25.88 (12.56-53.31)	**<0.0001**
Differentiation		
Grades 1-2	1.00	
Grades 3-4 (0-1.25y)	1.97 (1.40-2.76)	**<0.0001**
Grades 3-4 (1.25-5.0y)	0.79 (0.53-1.16)	0.231

Abbreviations CI=Confidence interval, HR=Hazard ratio

Table 11.E Cox multivariable analysis of relative risk of death from colorectal cancer within 5 years by CA19-9 expression

	HR (95% CI)	p-value
CA19-9 expression		
Low	1.0	
High	1.80 (1.09-2.98)	**0.023**
Age		
≤65	1.0	
>65	1.65 (1.01-2.72)	**0.047**
Gender		
Female	1.0	
Male	0.93 (0.57-1.53)	0.776
Stage		
Dukes A	1.0	
Dukes B	7.75 (1.02-59.09)	**0.048**
Dukes C	13.55 (1.81-101.31)	**0.011**
Dukes D	70.25 (9.51-518.77)	**<0.0001**
Differentation		
Grades 1-2	1.0	
Grades 3-4	1.09 (0.58-2.05)	0.793

Abbreviations CI=Confidence interval, HR=Hazard ratio

9. DISCUSSION

9.1. Tumour markers

9.1.1. Podocalyxin (PODXL)

Studies I and II show PODXL to be an independent marker in CRC of poor prognosis. The staining pattern of the two antibodies differed: by the mAb, patients with high cytoplasmic expression had a poor prognosis, whereas by the pAb, a similar phenomenon emerged for membranous PODXL expression. Correlations with clinicopathological parameters (except for tumour side) were similar, but interestingly, case-by-case expression of PODXL by mAb and pAb did not correlate. Compared to either antibody alone, combination of the results of both antibodies enlarged the group of patients with poor prognosis, and revealed a group with an even worse prognosis.

As it is an anti-adhesive molecule, aberrant PODXL expression has been suggested to support the disruption of cell-to-cell and cell-to-extracellular matrix adhesion, thus promoting tumour dissemination (144). Its ectopic expression correlates with increased invasion in breast and prostate cancer (191). In CRC, high cytoplasmic expression by the mAb and membranous expression by the pAb correlated with poor differentiation, advanced disease stage, and poor survival, in accordance with the literature (146,149).

Surprisingly, expression patterns varied, and case-by-case expression was not uniform between the two antibodies, even though they are known to recognise different epitopes within the extracellular portion of the PODXL molecule. As patients with concomitant high cytoplasmic PODXL expression by the mAb and membranous expression by the pAb had an even worse DSS than did those

with only membranous or only high cytoplasmic PODXL expression, it is possible that the two antibodies may describe a slightly different biological phase of PODXL in CRC. Possibly the polyclonal antibody recognises an active form of PODXL at the cell membrane, whereas the monoclonal antibody with its smaller target epitope is able to recognise overexpression of cytoplasmic PODXL, which either has a function in the cytoplasm, or is moving towards the cell membrane. Of every four patients with this concomitant positivity, nearly three had metastatic disease at diagnosis; this supports the role of PODXL overexpression in tumour-cell dissemination, later leading to metastases.

Another possibility is that these antibodies recognise different variants of PODXL; of the four protein-coding PODXL splice variants, the epitope sequence of the pAb matches three of them 100% (PODXL 001, 005, and 201; The Human Protein Atlas). The fourth splice variant matches 87% (PODXL 202). The epitope sequence of the mAb HES9 matches all splice variants 100%.

The difference that emerged between the two antibodies in relation to clinicopathological parameters was that high cytoplasmic expression by the mAb was more common in the right hemicolon (RHC) than in the left (LHC), whilst no such difference appeared with the pAb. The division between tumours by tumour location suggested by Bufill (192) is not based solely on anatomical site. It is based also on developmental differences: the RHC is derived from midgut and perfused by the superior mesenteric artery with a multilayered capillary network, whereas the LHC is derived from hindgut and perfused by the inferior mesenteric artery with a single-layer capillary network. A recent study by Yamauchi (193) suggests no discrete transition point at the splenic flexure, but a gradual change in histological and molecular characteristics from

ascending colon to rectum. This difference between expression patterns of the two PODXL antibodies may be due to differing cytoplasmic activity of PODXL in left- compared to right-sided tumours, but this requires further validation.

Both of the antibodies studied showed PODXL to be an independent marker in CRC of poor prognosis. No clear difference emerged between the two antibodies as prognostic markers, however, because their hazard ratios for 5-year risk of death were almost identical. Their prognostic roles and associations with clinicopathological parameters (except for tumour location for the mAb) corresponded with the literature's (146,149).

The differing expression patterns of the two antibodies offer a possibility for their combined use. A simple combination of the expressions created two new groups; one with low cytoplasmic/non-membranous and other with high cytoplasmic or membranous expression or both. The combination defined a larger number of patients with poor prognosis than did either antibody alone.

When combining the expression patterns into three new classes, we were able to identify a small group of patients with a grim prognosis. The size of this group was small, thus this phenomenon is of more biological interest than of clinical value.

9.1.2. REG4

Study IV showed that, in non-mucinous CRC, cytoplasmic REG4 expression associates with favourable clinicopathological parameters and that it is an independent marker of favourable prognosis in patients under 65.

In CRC, REG4 expression was higher in low-stage tumours and in those with mucinous histology. With mucinous tumours excluded, REG4 expression associated significantly with higher differentiation and low stage. REG4 expression also associated with MUC1, MUC2, and MUC5AC, which supports the finding that REG4-positive tumours are more highly differentiated than are REG4-negative tumours. No association between REG4 and neuroendocrine differentitation emerged. These results are in accordance with findings of Li et al (159) that REG4 IHC expression in CRC associates significantly with higher differentiation and with absence of venous invasion. Moreover, Li et al showed a trend-like association of REG4 expression with low T-stage, absence of lymph node metastasis, and local disease (Dukes A-B vs C-D). Similar results appear for gallbladder cancer, where positive REG4 IHC expression associates with higher tumour differentiation (194). Numata et al (158) reported controversial results for CRC: that higher REG4 mRNA expression associates with higher differentiation, deeper invasion (T-stage), lymphatic invasion, liver metastasis, and more advanced stage. They, however, measured mRNA levels by PCR, not by the actual protein expression.

It is interesting that higher serum levels of REG4 occur in many carcinomas than in healthy controls: in pancreatic (154), gastric (153) and gallbladder cancers (194), suggesting a potential use for serum REG4 as a diagnostic biomarker.

Study IV showed that REG4 IHC is a marker of favourable prognosis in non-mucinous CRC, but controversial results have also emerged; elevated tissue levels of REG4 mRNA in CRC may be a marker of poor prognosis (158). In CRC, Li et al. (159) and Zheng et al. (160) saw no prognostic role for REG4

IHC expression, but they did not analyze mucinous and non-mucinous cancers separately. In addition, antibodies, staining procedures, and analysis of stainings may have differed from those in Study IV.

REG4 expression is constitutively high in mucinous tumours such as pseudomyxoma peritonei and mucinous cystadenomas, and Study IV demonstrates that in CRC REG4, expression associates with markers of mucinous differentiation. This may explain why immunohistochemistry found no clear variation in REG4 expression in the group of mucinous CRC tumours, and why REG4 IHC expression was a marker of favourable prognosis only in non-mucinous CRC. It thus seems plausible that in CRC patients with poor prognosis, REG4 mRNA levels may be elevated, but this is not translated to protein. Further studies are warranted to compare REG4 mRNA levels with REG4 IHC case by case.

REG4 is expressed in inflammatory bowel diseases and also in the margins of peptic ulcers and is considered a marker of inflammation (104). In some whole-tissue sections, tumour tissue stained negative for REG4, but the adjacent benign epithelium expressed REG4 strongly, apparently representing an inflammatory reaction against the tumour.

In normal intestinal mucosa, high REG4 expression is apparent in the majority of entero-endocrine cells, with the co-expression of synaptophysin and chromogranin (104). Considering this fact, it is interesting that no association appeared between REG4 expression and neuro-endocrine differentiation.

Several reports suggest the oncogenic role of REG4 in the development of cancer in the gastrointestinal tract. The ultimate molecular mechanisms have, however, remained elusive. Bishnupari et al (195) reported that treatment of cultured colon adenocarcinoma cells with recombinant REG4 protein induced phosphorylation of the EGF (epidermal growth factor) receptor and Akt. They suggested that REG4 is a transactivator of the EGFR/Akt signaling pathway. A further elucidation of the role of exogenous REG4 as a regulator of cell growth potential is, however, awaiting identification of the putative REG4 receptor. No evidence shows that increased expression of REG4 by itself induces cancerous growth, however.

Regulation of REG4 expression is still poorly understood. REG4 is up-regulated in inflamed IBD mucosa and in IBD-like foci of gastritis-induced intestinal metaplasia in the stomach (104). This suggests that inflammatory cytokines may influence REG4 expression. Moreover, physiological REG4 expression is evident in cells with neuroendocrine differentiation, where REG4 co-expresses in neuroendocrine tumours with the neuronal transcription factor Hath-1 (atonal, Math-1) (151), one possible regulator of REG4.

9.1.3. N-glycosylation in colorectal cancer

Study III shows by MALDI-TOF MS analysis that the acidic and neutral N-glycan profiles of rectal adenomas and carcinomas clearly differ, and that principal component analysis of specific N-glycans can separate adenomas from carcinomas. Several specific N-glycan structure types were identified whose amounts in carcinomas either increase or decrease during adenoma-carcinoma transition or cancer progression. Differences in the amount of N-glycan

structures also appeared: between local and locoregional disease and between local and advanced cancer.

Sulphate esters are typical modifications of the digestive tract glycans (196) , and they were also apparent in previous glycosylation analyses of colorectal carcinoma and normal tissue samples (122). Sialylation, on the other hand, is more common in most tissues of the human body. Study III showed that N-glycan profiles of adenomas were complex and rich in the terminal glycan modifications typical of colon glycosylation. These included acidic esters partly replacing sialylation and also complex fucosylation of N-glycans. In comparison, in the acidic N-glycan profiles of the carcinomas, a drastic loss of rectal epithelial glycosylation features was apparent, including the acid ester modifications. Thus, the carcinomas showed dedifferentiation from the normal tissue-specific glycosylation, which was especially prominent at advanced stages. However, alongside dedifferentiation many glycan structures were increased in carcinomas, thus serving as potential novel cancer biomarkers.

Principal component analysis clearly showed the homogeneity of adenomas' N-glycan profiles and the difference between carcinomas' N-glycan profiles and those of adenomas, and differences among themselves. Similar differences appear between serous ovarian cystadenomas and serous carcinomas (Carpen, O et al, oral communication).

The serum N-glycomic profile differs in CRC patients from that in normal controls; adenoma patients' N-glycan profiles also differ from those of CRC patients (123). Seeing directly the changes in the glycomic profiles of tumour cells, however, requires MS analysis of the tissue, since changes in serum

glycosylation have been associated with altered glycosylation of immunoglobulins and of acute phase proteins (197,198).

Balog et al (122) reported an increase in pauci-mannocidic structures, sulphated N-glycans (acid esters), and sialyl Lewis-type epitopes in CRC samples compared to normal levels in control samples. Results in Study III were in accordance in regards to pauci-mannosidic structures and glycan signals indicating sialyl-Lewis type structures, but the amounts of sulphated N-glycans were lower in carcinomas than in adenomas. Balog et al. had studied tumours originating from all of the colorectum, whereas tumours in Study III were of rectal origin; they also studied the difference between carcinoma tissue and adjacent normal tissue, but did not study adenomas. At a general level, results in Study III corresponded with those of Balog et al., a sign of this method's repeatability.

MS allows the identification of a large number of glycan structures whose expression differs between carcinomas and adenomas; these included increased sialylated and pauci-mannose structures. Moreover, differences between local carcinomas and metastasized tumours were visible. These glycan structures are potential tumour markers that should be studied in large patient series. Study III analyzed two candidate glycan: sLe_a, better known as serum tumour marker CA19-9, and pauci-mannose. High tissue expression of sLe_a has been correlated with poor prognosis and unfavourable clinicopathological parameters in CRC (199-201). Results of Study III are in accordance with these findings. Elevated expression of pauci-mannose N-glycans correlated with non-mucinous histology, and in advanced CRC it associated with poor prognosis. Pauci-mannosidic structures have also been associated with lysosomal glycoproteins,

but the mechanism of their accumulation in malignant tumours is currently unknown. In addition to strong intracellular staining with the pauci-mannose antibody, we detected membraneous accumulation of these structures in cancer cells, which suggests the potential utility of this glycan group as a source of novel cancer-associated antigens.

Because blood-group antigens are built of glycan structures, only patients with blood group A Rh+ were included in Study III for MS, to eliminate possible blood group influence. However, no signals in relation to this blood-group were observable, so it is possible that in future studies the influence of blood groups can be disregarded.

The majority of glycosylation differences appeared between adenomas and carcinomas, but differences appeared also between local and more advanced carcinomas, differences that may reflect tumour ability to invade and metastasize. Most colorectal carcinomas arise from adenomas by accumulating mutations in key tumour-suppressor genes and oncogenes in a slow process taking from years to even decades. Major protein glycosylation is already undergoing a shift from strictly tissue-specific N-glycan structures in adenomas into dedifferentiated glycosylation in early-stage carcinomas. Further studies are essential to study the possible role of such changed glycosylation in carcinoma invasion and metastasis.

Study III indicates that in MS glycosylation analysis, a distinction is apparent between benign and malignant tumours. Based on PCA results, it is clear that a complex MS profile can be transformed into a simple equation, predicting

malignancy. A prospective study with a larger sample cohort should analyze the MS method's sensitivity and specificity.

Since both MS and data analysis can be automated, MS alone could prove clinically useful. The current price-tag for analysis of a N-glycan profile of one tissue sample ranges around 1000 €, but with automation and a sufficient number of samples to be analyzed, the cost would fall to around 100 € per sample, making it competitive with traditional diagnostic methods. Biotechnological companies proposed a comparable serum proteomics method for clinical use at a similar price over a decade ago, but its insufficient sensitivity and specificity have remained a problem.

In addition to pure MS analysis as a diagnostic tool, transforming differences in N-glycosylation found by MS into immunohistochemical or serum biomarker assays would prove useful. These methods require specific antibodies, which can either be produced or be sought in peptide libraries.

9.2. Strengths and limitations of study materials and methods

The TMA technique used in all four studies allows analysis of large patient cohorts, but allows analysis of only a small proportion of the tumour, which, considering intra-tumoural heterogeneity, could cause misinterpretation. However, with adequate multiple sampling from histologically representative areas, TMA leads to results that are in accordance with those from whole-tissue sections (187,202,203).

The large patient cohort comprising almost 850 CRC patients consecutively operated on in HUH between 1983 and 2001 is well characterised

clinicopathologically, and the survival data were comprehensively gathered. This patient cohort has a long follow-up, but using old patient data has its limitations. Instead of today's widely used TNM UICC, the modified Dukes stage according to ACPS was used, because it was used at HUH during the years of these patients' primary treatment. Survival rates have also improved over time, due to improvements in surgical techniques, pathological staging, preoperative imaging, and oncological treatments. The number of lymph nodes has increased, with now a minimum of 12 lymph nodes studied and examined, leading to stage migration from stage II to stage III (204-206). This has led to improved prognosis in stage II as a consequence, and has also improved prognosis in stage III (often called the Will-Rogers phenomenon). In addition to leading to more frequent adjuvant therapies, stage migration has an impact on overall prognosis in CRC.

In Study III, the number of tumour samples was relatively low, since analysis of the full N-glycan profile of tumour samples was both time-consuming and laborious. Possible variance in glycosylation resulting from tumour location was eliminated by including only tumours of the rectum. As blood-group antigens are built of glycan structures, only patients with blood group A Rh+ were included, to eliminate any possible influence. Such strict inclusion criteria allowed the use of a reasonably small sample cohort to identify significant glycosylation changes apparently related to carcinoma progression.

9.3. Concluding remarks

As colorectal cancer and other cancers are a growing burden in modern health care, all advances in prevention, diagnostics, and treatments are valuable. Novel biomarkers are essential to detect cancer earlier and to identify patients for

targeted and individualized therapy. Before drawing conclusions regarding the effect of a specific biomarker on individualized treatment, the biomarker's prognostic and predictive role must be thoroughly validated in sufficiently large prospective clinical trials (207).

This study confirms PODXL to be a marker of poor prognosis in CRC by means of two antibodies, each of which recognized its own group of patients with a poor prognosis. Combination of the two PODXL antibodies defined a larger number of patients with poor prognosis and also a small group of patients with an even worse prognosis. A trial combining both of the PODXL antibodies with PODXL gene-mutation information would be interesting and would further clarify the biological function of PODXL. Study IV showed that REG4 IHC expression to be a marker of favourable prognosis in non-mucinous colorectal cancer. Although these results are in disagreement with those obtained by evaluating mRNA levels; our discrepancies with others' findings warrant further studies.

Study III showed that rectal adenomas can be identified from carcinomas based on MS analysis of their N-glycan profile. Glycosylation differences existed also between local and more advanced carcinoma. In the future carcinoma-related glycan structures identified in this study can be tested as potential prognostic biomarkers by detection of these glycan structures in situ by immunohistochemistry. Changes in glycosylation profile can evolve into a simpler equation that predicts malignancy, requiring validation in larger series.

10. CONCLUSIONS

- PODXL was associated with unfavourable clinicopathological parameters and was an independent marker of poor prognosis in colorectal cancer.
- The two PODXL antibodies studied recognised different groups of patients, both with poor prognosis. Combined use of the antibodies revealed a group with even worse prognosis.
- REG4 IHC expression was associated with favourable clinicopathological parameters and was an independent marker of better prognosis in non-mucinous CRC.
- N-glycan profiles or rectal adenomas and carcinomas differed.
- MS analysis of N-glycan profiles can identify specific glycan structures useful for creating antibodies for immunohistochemistry.

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